Molecular Analysis of WEHI-3B JCS Myeloid Leukemia Cell Differentiation Induced by Biochanin A and Midazolam

by

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Statement

All the experimental work reported in this thesis was performed by the author, unless otherwise specified.

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Leukemic cell differentiation is a very complex process which one can never work out by his own. Each of us works on a small piece making a contribution to the whole picture. The field is full of speculations and unknowns which make our work particularly exciting and challenging. In addition, it allows one's association and imagination. Throughout this two year, I am greatly indebted to my supervisor Dr. M.C. Fung for getting me into the field. I am also very grateful to his patience, tolerance, stimulating criticism and encouragement, not to mention his care and technical assistance. A special thanks must also go to my thesis committee, Dr. H.S. Kwan and Dr. V.E.C. Ooi, for their care and for keeping me going. I also wish to express my gratitude to Dr. K.K. Mark for his interest and enthusiastic support on everything I have been doing. I would also like to thank Dr. N.K. Mak, at the Hong Kong Baptist University and Dr. K.N. Leung at the Department of Biochemistry, CUHK who have followed my project closely and gave appropriate suggestions through my supervisor.

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Abbreviations

The following abbreviations will be used without explanations :

Abbreviation	Full term
A	Adenine
AGM	Aortic/gonad/mesonephros region
ALF	Automatic Laser Fluorescent system
ALP	Alkaline phosphatase
AML	Acute myeloid leukemia
Amp^{100}	Ampicillin, 100 μg/ml
AMV	Avian myeloblastosis virus
AP-1	Activator protein-1
ara-C	1-β–D-arabinofuransyl-cytosine (cytosine arabinofuranoside)
Bas-CFC	Basophils-colony-forming cells
BFU-E	Burst-forming units-erythroid
BLAST	Basic Local Alignment Search Tool
bHLH	Basic helix-loop-helix
bHLH-Zip	Basic helix-loop-helix leucine zipper
BM	Bone marrow
bp	Base pair(s)
C	Cytosine
CBR	Central benzodiazepine receptor
CD	Cluster of differentiation (e.g. CD34)
cDNA	Complementary DNA
CFC-mixed	Colony-forming cells-mixed
CFU-A	Colony-forming units-agar
CFU-Bl	Blast colony-forming units
CFU-C	Colony-forming units-culture
CFU-E	Colony-forming units-erythroid
CFU-GEMM	Colony-forming units-granulocytes, erythrocytes, macrophages and megakaryocytes
CFU-GM	Colony-forming units-granulocytes and macrophages
CFU-Meg	Colony-forming units-megakaryocytes
CFU-S	Colony-forming units-spleen
CFU-T	Colony-forming units-T cells
CML	Chronic myeloid (myelogenous) leukemia
CMMoL	Chronic myelomonocytic leukemia
CRU	Competitive repopulation unit
CSF	Colony-stimulating factor
CSPD	Disodium 3-(4-methoxyspirol{1,2-dioxetane--3,2'-(5'-chloro)tricyclo[3.3.1.1$^{3.7}$]decan}-4-yl) phenyl phosphate
dATP	2'deoxyadenosine 5'-triphosphate
dCTP	2'deoxycytidine 5'-triphosphate
DDBJ	DNA Data Bank of Japan
ddH_2O	Double distilled water
ddNTP	2',3'-dideoxyribonucleotide triphosphate
DDRT-PCR	Differential display
DEPC	Diethyl pyrocarbonate
dGTP	2'deoxyguanosine 5'-triphosphate
DIG	Digoxigenin
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acids
dNTPs	Deoxyribonucleoside triphosphate

dpc	Days postcoitum
DTT	Dithiothreitol
dTTP	2'deoxythymidine 5'-triphosphate
E_2	17β–Estradiol
EDTA	Ethylenediamine-tetraacetic acid
EF-1α	Elongation factor
EMBL	European Molecular Biology Laboratory
Eos-CFC	Eosinophils-colony-forming cells
Epo	Erythropoietin
ES	Embryonic stem cells
FGF	Fibroblast growth factor
FTP	File transfer protocol
G	Guanine
GABA	γ–aminobutyric acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
G-CFU	Granulocyte-colony-forming units
G-CSF	Granulocyte colony-stimulating factor
GM-CFC	Granulocytes and macrophages-colony-forming cells
GM-CSF	Granulocyte and monocytes-colony-stimulating factor
HAP	Hydroxylapatite column chromatography
HIM	Hematopoietic inductive microenvironment
HLA	Human leukocytes antigen
HPRT	Hypoxanthine phosphoriboxyltransferase
HPP-CFC	High proliferative potential colony-forming cells
HSC	Hematopoietic stem cells
IAP	Intracisternal A particle
IFN	Interferon
IL-	Interleukin- (e.g. IL-3, interleukin-3)
IL-1RtI	IL-1 receptor type I
IL-1RtII	IL-1 receptor type II
IPTG	Isopropyl-β–D-thio-galactopyranoside
JCS	WEHI-3B JCS cells
kb	Kilobase
LCM	Lung-conditioned medium
LF	Lactoferritin
LIF	Leukemia inhibitory factor
LPS	Lipopolysaccharide
LTC-IC	Long term culture initiating cells
LTRC	Long term repopulating cells
LTR-HSCs	Long term repopulating-hematopoietic stem cells
M-CSF	Monocyte-colony-stimulating factor
Meg-CFC	Megakaryocytes-colony-forming cells
M-MLV	Molony murine leukemia virus
MOPS	Morpholinopropanesulfonic acid
MPO	Myeloperoxidase
mRNA	Messenger RNA
MT	Mouse transcript sequence
NBT	Nitroblue tetrazolium
NCBI	National Centre for Biotechnology Information
NIH	National Institute of Health
O.D.	Optical density
P_4	Progesterone
PBR	Peripheral benzodiazepine receptor
PCI	Phenol-chloroform-iso-amyl alcohol
PCR	Polymerase chain reaction
PG	Prostaglandins
PGE_2	Prostaglandin E_2
PHSC	Primitive/pluripotent hematopoietic stem cells
PLSC	Pluripotent lymphoid stem cells

PPSC	Pluripotent stem cells
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear cells
PTK	Protein tyrosine kinase
PTx	Pertussis toxins
-R	Receptor (e.g. LIF-R, LIF receptor)
RA	Retinoic acid
RAP-PCR	RNA fingerprinting by arbitrarily-primed PCR
RNA	Ribonucleic acid
rpm	Revolution per minute
RPMI 1640 medium	Roswell Park Memorial Institute Tissue Culture Medium 1640
rRNA	Ribosomal RNA
RT-PCR	Reverse-transcription polymerase chain reaction
SCF	Stem cell factor
SDS	Sodium dodecyl sulfate
SSC	Standard saline citrate
ssDNA	Single-stranded DNA
T	Thymidine
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TGF	Transforming growth factor
THSC	Totipotent hematopoietic stem cells
Tm	Melting temperature
TNF	Tumor necrosis factor
TPA	12-O-Tetradecanoylphorbol-13-acetate
U	Unit
UV	Ultra violet
Vit D_3	Vitamin D_3
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

All genes described in the text will be written in small italic letters e.g. c-*myb* and proteins will be written in capital block letters e.g. MYB.

Abstract

WEHI-3B (JCS) cells could be induced to differentiate by either biochanin A or midazolam. While biochanin A led JCS cells to follow an entire monocytic pathway, midazolam drove the cells to produce both macrophages and granulocytes.

Using reverse-transcription polymerase chain reaction (RT-PCR) followed by Southern/dot blot hybridization, cytokine and cytokine receptor gene expression profiles were revealed during the biochanin A- and midazolam-induced JCS cell differentiation. In particular, IL-1α and IL-1β were induced after incubation with biochanin A for 46 hours. In contrast, the expression of IL-1α remained undetected and that of IL-1β was decreased after midazolam treatment. TNF-α was slightly up-regulated in biochanin A- but not midazolam-treated JCS cells. The expression of LIF was increased significantly at 46 hours by biochanin A but unchanged by midazolam. Unlike the above genes, the expression levels of LIF-R and IL-4 were essentially undetectable during both biochanin A- and midazolam-induced differentiation.

Differential gene expression during the midazolam-induced JCS cell differentiation was investigated by a modified protocol of RNA-fingerprinting by arbitrarily-primed polymerase chain reaction (RAP-PCR). Two rounds of cDNA probe screening were employed to eliminate 'false positives', genes that were falsely taken as differentially expressed genes. A total of 142 differentially amplified fragments were obtained from the original fingerprints. After the second round of screening, only 12 fragments suggestive of differential expression were selected for study. Eight of these fragments (genes) were later confirmed to be differentially expressed in the midazolam-induced cells. One of the fragments contained a stretch of sequence aligned completely with the MT sequence repetitive element; two others aligned with mRNA sequences of glucocerebrosidase and ribosomal protein S25. The remaining five fragments had no significant homology with the reported sequences in the Genbank and are putative novel genes.

The expression pattern of the isolated genes in midazolam-induced JCS cells can be classified into four types : (1) down-regulation at 5 hours (three genes); (2) steady down-regulation (two genes); (3) transiently up-regulation at 1 hour (one gene) and (4) gradual up-regulation (two genes). Interestingly, all these genes have a different expression pattern in the biochanin A-induced JCS cells as consistent with the study on cytokine and cytokine receptor gene expression. These differences in gene expression, including those of cytokine and cytokine receptor genes could be inducer-specific and/or lineage-specific.

The expression profiles of these isolated genes during embryonic development were also studied using RT-PCR. Some of these genes were specifically up-regulated at 9.5, 11.5 and 17.5 days postcoitum (dpc). These time points coincided with the time of early organogenesis/hematopoietic precursor cell activity in the aortic-gonad-mesonephros (AGM) region (9.5 dpc), organogenesis/fetal liver hematopoiesis (11.5 dpc) and late fetal growth/bone marrow hematopoiesis in the fetus (17.5 dpc). Roles of these genes in the development of hematopoietic tissue and/or non-hematopoietic tissue of the embryo were suggested.

Direct strategies are proposed to determine the exact function of these isolated genes in both myeloid cell differentiation and embryonic development. Hopefully, such studies, together with those done by others, would allow a greater appreciation of the hematopoietic system and perhaps, novel treatments for leukemia, based on restoring normal cell differentiation and maturation are possible very soon.

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Appendix

Chapter One General Introduction

Cell differentiation is an indispensable process in hematopoiesis. To appreciate the importance of studying the process, a prior understanding of the hematopoietic system as well as some basic concepts of cell differentiation are necessary.

1.1 Hematopoiesis

1.1.1 Ontogeny of the hematopoietic system

Hematopoiesis is commonly known as blood cell formation. It is an ordered process in which stem cells differentiate into mature blood cells through sequential divisions. The primary hematopoietic sites differ at different time during the development (Figure 1.1). In mouse, the first hematopoietic cells are derived from the blood islands (hemangioblast) of the yolk sac and the process is known as primitive or embryonic hematopoiesis. The detectable cells are mainly primitive nucleated erythrocytes and progenitors (CFU-C) capable of producing granulocytes and macrophages (Moore and Metcalf, 1970). Later, T- and B-lymphoid activities can be detected (Liu and Auerbach, 1991). Cells from the yolk sac then migrate to the fetal liver building up a large population and the process is known as the first wave of colonization (Moore and Metcalf, 1970). Stem cells, progenitor cells, embryonic erythroid cells, macrophages and B cells can then be found in the liver. Migration of progenitors (CFU-S) from the aortic/gonad/mesonephros region (AGM region) to the fetal liver makes up the second wave of colonization (Dzierzak and Medvinsky, 1995; Medvinsky *et al.*, 1993). The colonization results in stem cell (LTR-HSCs) and progenitor activities (CFU-S) detectable in the liver (Medvinsky *et al.*, 1993; Dzierzak and Medvinsky, 1995). Definitive hematopoiesis is fully established in the liver after two waves of colonization and population of cells into the developing spleen and bone marrow of the fetus starts. Shortly before birth and thereafter, the bone marrow becomes the major site of hematopoiesis (95%). However, both splenic

and liver hematopoiesis can resume active if the bone marrow is diseased or the demand for blood cells is extreme during adulthood (extramedullary hematopoiesis).

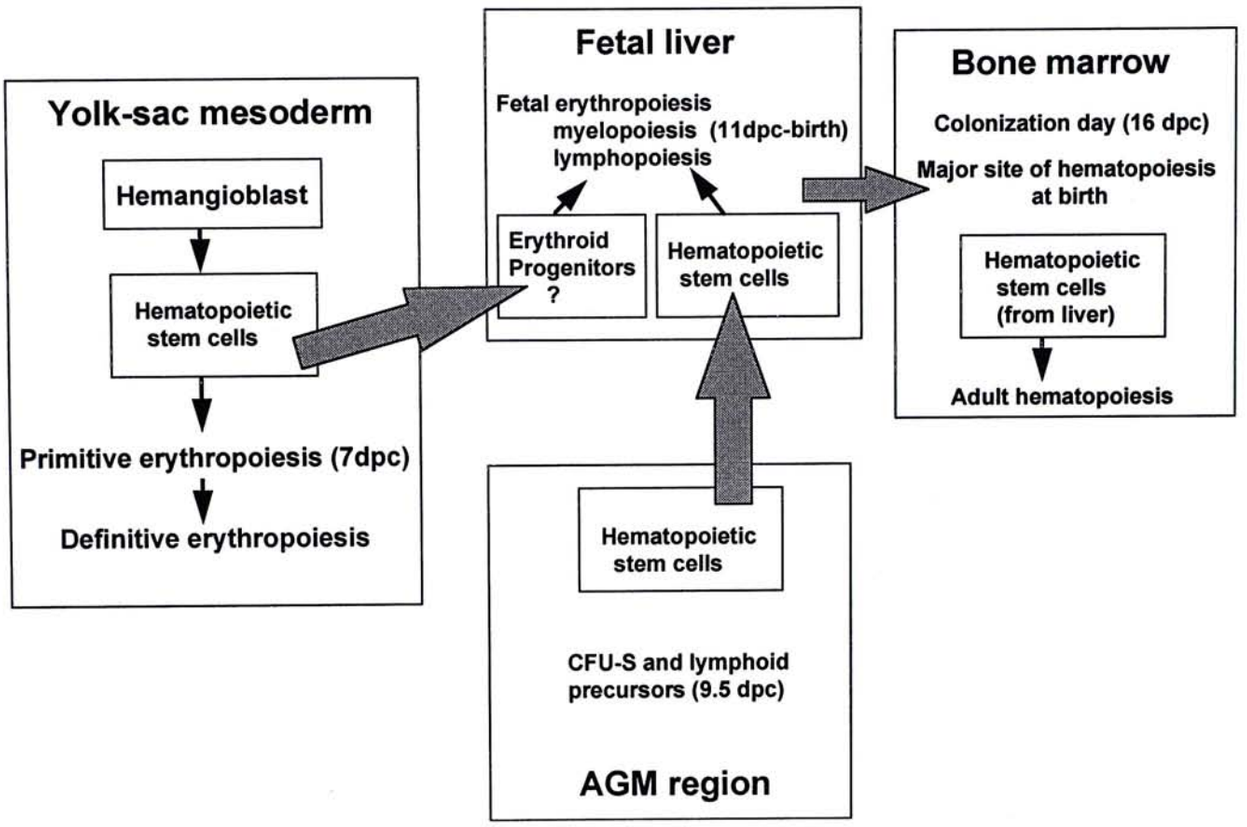


Figure 1.1. **The ontogeny of the hematopoietic system (mouse).** The large boxes represent the different hematopoietic organs during mouse development. The dark thin arrows represent cell differentiation. The large gray arrows indicate cell migration. Primitive hematopoiesis occur at around 7 dpc (days post coitum) in the yolk sac, CFU-S and lymphoid precursors can be detected in the AGM region at 9.5 dpc. Fetal liver hematopoiesis starts at 11 dpc and continue until birth. After birth, the main site of hematopoiesis switches to the bone marrow. The first wave of colonization involves cells (thought to be committed erythroid progenitors) moving from the yolk sac to the liver while cells move mainly from AGM region to the liver in the second wave of colonization (adapted from Paulson and Bernstein, 1995).

1.1.2 Hierarchy of hematopoietic cells

Although the maturing hematopoietic cells are not arranged in distinct layers and apparently situate in a random manner in the bone marrow and in the spleen, segregation of granulocytes and erythroid cells in the mouse marrow was observed (Lord and Wright, 1984). Moreover, stem cells seem to be concentrated at the periphery of the mouse marrow cavity (Metcalf, 1988) with blood cell precursors occupying a specific position (Pallister, 1994). Megakaryocytes are located adjacent to the venous sinus with their cytoplasmic processes projecting into the lumen of the sinus while red cell precursors lie adjacent to the venus sinus in erythroblastic islands. Granulocyte and monocyte precursors, however, tend to lie more deeply within the blood forming cavity.

The maturing cells of each cell lineage in the hematopoietic tissue can be identified by their characteristic morphology and arranged into likely maturation sequences. Three major differentiation compartments (compartment for stem cells, progenitor cells and maturing cells) can be distinguished by using a number of clonal-analytical procedures (Figure 1.2). Cell population of each compartment is derived from cells of the preceding one and the cells gradually decrease in gene activity for proliferation and increase in gene activity for differentiation as they move from one compartment to the other. As a result, the cells lose their self-renewal capacities and display the antigenic, biochemical and morphological features specific to mature cells of the appropriate lineages.

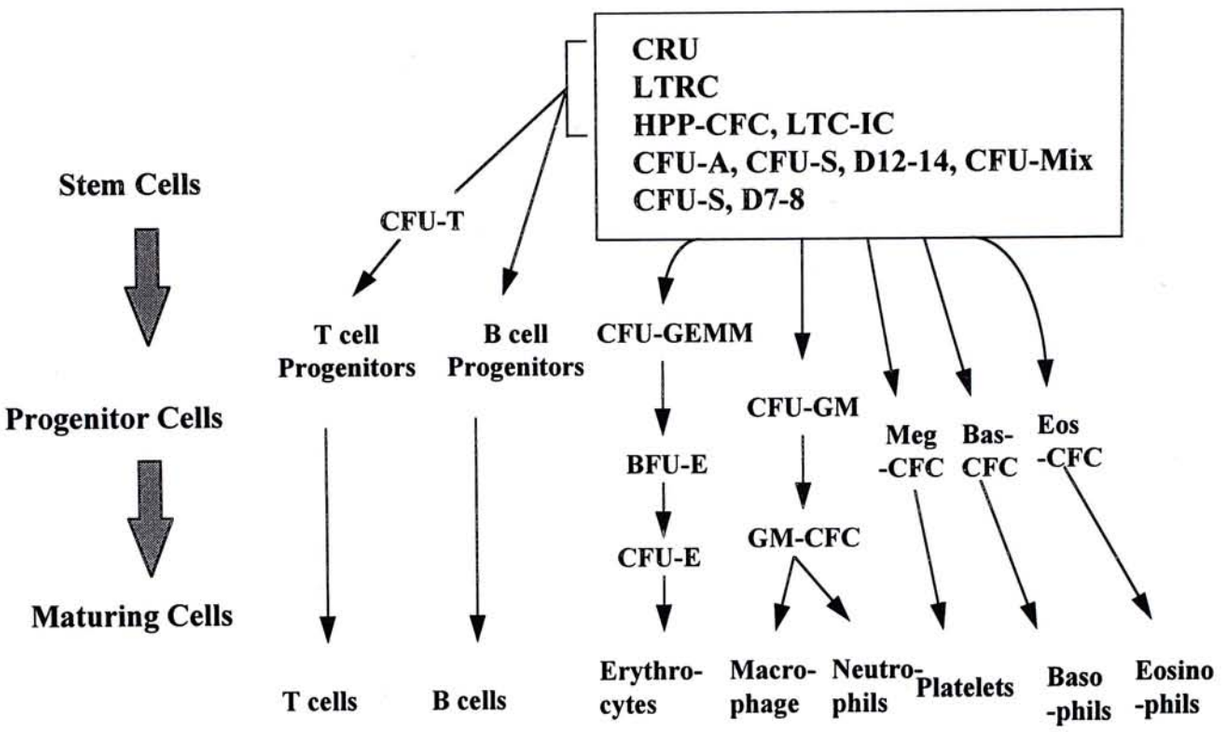


Figure 1.2. **A simplified diagram showing the hierarchy of hematopoietic cells.** Three differentiation compartments can be identified : the stem cells, progenitor cells and maturing cells. Each of these compartment is very heterogeneous. The box shows the different description of stem cells by various clonogenic assays. The progenitors are also defined by different assays as either colony-forming units (CFU), burst-forming units (BFU) or colony-forming cells (CFC). The stem cells are able to self-renew and give rise to various committed progenitors before differentiating into the numerous mature blood cells. The intermediate progenitors for lymphoid lineage (T and B cells) are omitted in the diagram. (Gunji and Suda, 1995; Metcalf, 1988; Metcalf and Nicola, 1995; Tsukamoto *et al.,* 1995). Abbreviations : CRU, competitive repopulation units; LTRC, long term repopulating cells; HPP-CFC, high proliferative potential colony-forming cells; LTC-IC, long term culture initiating cells, CFU-A, colony-forming units-agar; CFC-mixed, colony-forming cells-mixed; CFU-S, D12-14, colony-forming units-spleen, day 12-14; CFU-Bl, blast colony-forming units; CFU-S, D7-8, colony-forming units-spleen, day 7-8, CFU-T, colony-forming units-T cells; CFU-GEMM, colony-forming units-granulocytes, erythrocytes, macrophages and megakaryocytes; BFU-E, burst-forming units-erythroid; CFU-E, colony-forming units-erythroid, CFU-GM, colony-forming units-granulocytes and macrophages, GM-CFC, granulocytes and macrophages-colony-forming cells; Meg-CFC, megakaryocytes-colony-forming cells; Bas-CFC, basophils-colony-forming cells, Eos-CFC, eosinophils-colony-forming cells.

Stem cells

Stem cells occur at approximately 1 per 10^5 to 1 per 10^6 marrow cells. These refer to only about 0.2 % of the hematopoietic cells (Metcalf, 1988). Stem cells are self-renewable and multi-potential. They can produce progenies identical to themselves in appearance and differentiation potential and yield mature blood cells of all types. They are also remarkable in proliferative potential and a larger progeny population of lowered multi-lineage differentiation potential and self-renewal ability are produced. Despite their distinct self-generation and differentiation potentials, most of the stem cells are not in cell cycle (G_0 phase) and divide at a slow rate *in vivo*. Only 18-22 % of them are usually in the active phase of the cell cycle ($S/G_2/M$ phase) (Paulson and Bernstein, 1995).

Stem cells have been defined in various clonogenic assays aiming at measuring their long-term repopulating ability and maximal differentiating ability. Because of the heterogeneity of the stem cell population, stem cells are operatively known as :

- *in vivo* competitive repopulation units (CRU),
- *in vitro* long term repopulating cells (LTRC),
- high proliferative potential colony-forming cells (HPP-CFC),
- long term culture initiating cells (LTC-IC),
- colony-forming units-agar (CFU-A),
- colony-forming cells-mixed (CFC-mixed),
- colony-forming units-spleen, day 12-14 (CFU-S D12-14),
- blast colony-forming units (CFU-Bl)
- colony-forming units-spleen, day 7-8 (CFU-S D7-8)

arranged approximately in their ascending order of maturation (Figure 1.2) (Gunji and Suda, 1995; Metcalf, 1988; Metcalf and Nicola, 1995; Tsukamoto *et al.*, 1995).

Stem cells are difficult to characterize morphologically. Generally speaking, they have large nucleus to cytoplasm ratio and prominent nucleoli inside the nuclei. The cytoplasm is not basophilic and contains no granules. The most primitive human stem cells are identified by being $CD34^+$, Thy-1^+ and $Rh123^{lo}$. They are also $CD71^-$, $HLA\text{-}DR^-$, $CD45RA^-$, $CD45RO^+$, $CD38^-$, $CD33^-$, c-*kit* ^{lo} and Lin^- (Tsukamoto *et al.*, 1995; Weissman, 1995). Similarly, primitive stem cells are recognized by Thy^{lo}, $Lin^{-/lo}$ and $Sca\text{-}1^+$ in mouse (Spangrude *et al.*, 1988). Distinct intermediate forms of stem cells can also be distinguished from each other by the stage-specific expression of cell surface markers and their dependence on growth factors.

Stem cells are recognized as hematopoietic stem cells (HSC), primitive/pluripotent hematopoietic stem cells (PHSC), pluripotent lymphoid stem cells (PLSC), pluripotent stem cells (PPSC/PSC) and totipotent hematopoietic stem cells (THSC).

Progenitor cells

Progenitor cells represent 1 % of the hematopoietic cells and occur at about 2-5 per 1000 marrow cells (Metcalf and Nicola, 1995). They are a transit population formed by stem cells during the production of maturing progeny. Each of the progenitor cells is capable of generating up to 10^5 maturing progeny. The cells are committed having restricted differentiation capacity. They usually go to a single lineage of differentiation but bi- and tri-lineage-committed progenitor cells may also exist. When the cells become committed to a certain differentiation lineage, the specific genetic programme for that lineage becomes activated. This commitment is irreversible so that progenitor cells cannot switch to another lineage or revert to multi-potential stem cells in general. Progenitor cells appear to have minimal or no capacity for self-generation *in vitro.* However, they are usually in cell cycle giving rise to colonies of maturing progeny in response to hematopoietic growth factors *in vitro.* The progenitor cell population is heterogeneous. For example, progenitor cells in the erythroid lineage can be further subdivided into the erythroid burst forming units (BFU-E) and the erythroid colony-forming units (CFU-E) with BFU-E being less

mature. Granulocytes progenitors can also be arranged into granulocyte progenitor cells (CFU-GM) and cluster-forming cells (GM-CFC) with the latter being more mature.

Progenitors cells were extensively studied by clonogenic culture system and have been described as colony-forming units :

- CFU-GEMM (colony-forming units-granulocytes, erythrocytes, macrophages and megakaryocytes),
- BFU-E (burst-forming units-erythroid),
- CFU-E (colony-forming units-erythroid),
- CFU-GM (colony-forming units-granulocytes and macrophages),
- CFU-M (colony-forming units-macrophages),
- CFU-G (colony-forming units-granulocytes),
- CFU-Eo (colony-forming units-eosinophils)
- CFU-Meg (colony-forming units-megakaryocytes).

Progenitor cells are large mononuclear, blast-like cells with agranular basophilic cytoplasm. Alternatively, they can be identified by expression of specific lineage markers.

Maturing cells

The dividing and maturing populations are the majority of cells found in the hematopoietic tissues. They are the progeny of progenitor cells and form a recognizable sequence of cells by morphology within each lineage. The least mature of these cells have a considerable capacity for proliferation yielding about 2-200 colonies in total *in vitro* (Metcalf, 1988). With progressive maturation, the cells gradually lose the proliferative potential and eventually mature to a postmitotic stage, after which no further cell division is possible generally.

A functional blood system consists of a minimum of eight distinct cell types : T-cell, B-cell/plasma cell, macrophage/monocyte, neutrophil, erythrocyte, megakaryocyte/platelet, mast cell and eosinophil. With regard to our special interest, the myeloid lineage, successive intermediates derived from granulocyte-macrophage progenitor cells can be recognized in increasing maturity (Figure 1.3). In granulocyte formation (granulopoiesis), the myeloblast is the earliest recognizable stage. Myeloblasts give rise to promyelocytes characterized by their content of azurophilic granules (primary granules). From the promyelocyte stage onwards, the relative proportion of primary granules progressively decreases concomitant with an increase in specific (secondary) granules. From the myelocyte stage through the metamyelocyte stage to mature granulocyte (neutrophils), the nucleus becomes increasingly segmented and the nucleus to cytoplasm ratio decreases. The immediate precursor of mature granulocyte is called stab cell or band form which has an irregular horseshoe or ring-shaped nucleus. In monocyte formation (monopoiesis), the monoblast is the only recognizable precursor of the monocyte. Promonocyte, with round to oval nucleus is rare and difficult to be distinguished. Maturing monocyte has a progressively indented nucleus and increases in azurophil granules. It is also smaller than promonocyte and circulates in blood for one or two days before migrating into tissues as macrophage (Wheater *et al.,* 1987; Krause and Cutts, 1994) (Figure 1.3).

Besides morphological changes, both granulopoiesis and monopoiesis can be traced by following some antigenic, cytochemical or biochemical evidence. Antigenic markers like Mac I (CR3, CD11b), CD16 and cytochemical markers like chloroacetate esterase are expressed upon maturation of both granulocytes and monocytes. C_3R, FcR, F4/80 and M-CSFR are early markers of macrophage differentiation while lysozyme is a late marker. Myeloperoxidase (MPO) lactoferritin (LF), alkaline phosphatase (ALP), defensin, cathepsin G, G-CSFR and Gr-1 are all markers for granulocytic differentiation. J11d decreases in expression during monocytic differentiation but increases in expression during granulocytic differentiation (Fung *et al.,* 1992). Biochemical assays for non-specific esterase is specific for macrophage and granulocyte differentiation while NBT reduction assay is designed for early monocytic and granulocytic differentiation. As mature granulocytes switch to the

utilization of glycolytic pathway for ATP production, lactate dehydrogenase activity can also be a marker of mature granulocytes.

Finally, it is worth mentioning that to divide the hematopoietic cell population into discrete compartments in the above discussion may have over-simplified the hierarchies of these cells. In fact, hematopoiesis is likely to involve a continuous change rather than clear-cut steps. Thus, the stem/progenitor cell hierarchy may exist as a spectrum of cell populations having a continuum of differentiation potentials and self renewal capacities (Till, 1976; Lemischka, 1992).

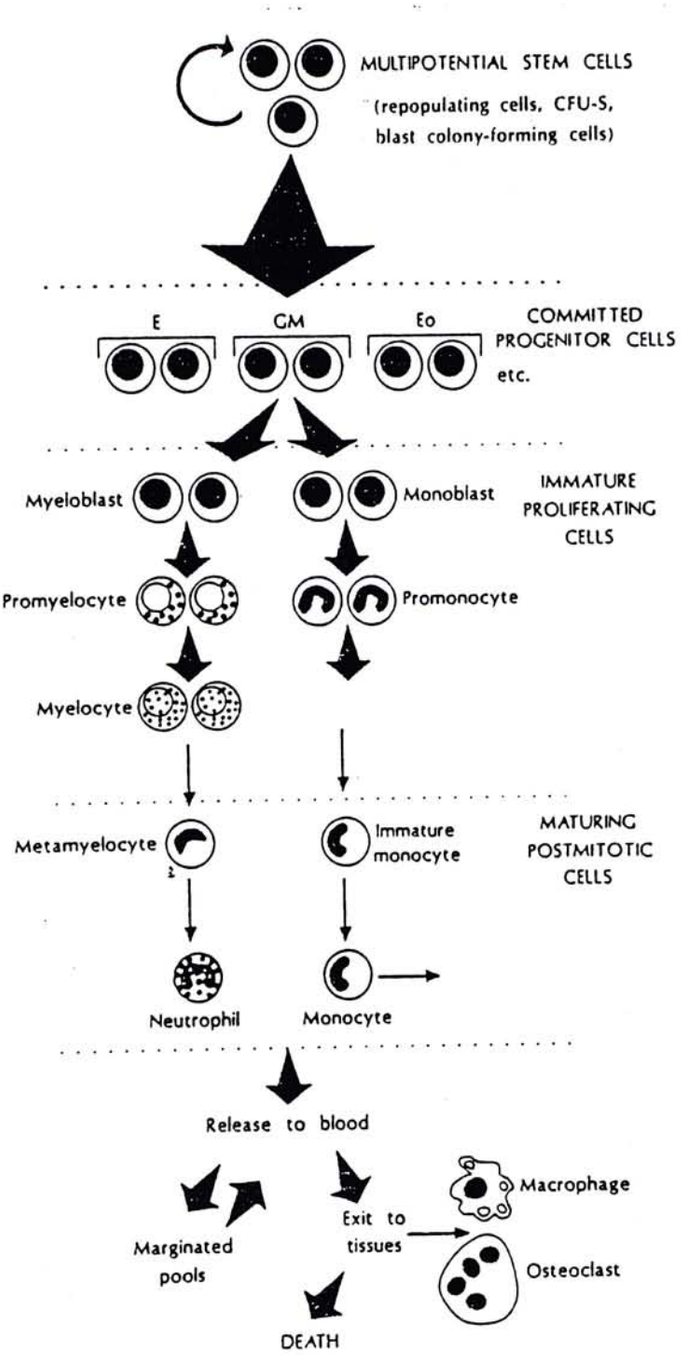


Figure 1.3. **Schematic diagram of the major stages by which mature granulocytes and monocytes are generated.** E denotes erythroid progenitor cells; GM, granulocyte-macrophage progenitor cells, Eo, eosinophil progenitor cells. (reproduced from Metcalf and Nicola, 1995)

1.1.3 Characteristics of a functional blood system and the need for regulation

There are several unique features of the blood system that make it different from other tissues in the body. The blood system is composed of at least eight very different mature blood cells modified to fit for their own functions. These cells are dispersed throughout the body. They have limited life span ranging widely from several hours (granulocytes) to weeks (erythrocytes) or years (memory cells). While the cells would finally die, they have gradually lost their ability to reproduce in the course of differentiation and maturation. In addition, as the whole system turns to the stem cells in the bone marrow for ultimate and continuous production of all blood cells, no more *de novo* hematopoiesis occurs after the liver definitive hematopoiesis has established.

Hematopoietic regulation is necessary to maintain the required cell number and cell types in the system so as to meet the demands of the body at different situations. That is, the system needs not only to produce basal level of blood cells for daily consumption but also to respond at emergency (e.g. bleeding and pathological conditions). Such regulation would only be effective, however, if the distinct features of the blood system are also taken into considerations. Since no more *de novo* stem cells would be produced in adult, the 'already-present' stem cell population must be self-sustaining without being exhausted. The cell production rate is also important as different cell types have different half-lives and require different specific rates of renewal. At the same time, the degree of differentiation of each cell type must be kept up for proper function. As the hematopoietic tissues are dispersed widely, there must be some effective mechanisms to detect the needs of the body and blood cell production of the correct lineages can be carried out in the bone marrow.

To suffice an effective regulation, various control points are monitored by different hematopoietic regulators and some pre-determined steps or programs are followed. For the arrangement of the hematopoietic subpopulations, (1) cell proliferation, (2) self-generative versus differentiative cell divisions, (3) differentiation commitment induction as well as (4) initiation and completion of

maturation are under control. In view of cell function, (1) regulation on the release of mature cells, (2) discharge of certain cell types to the tissues, (3) functional activation, (4) maintenance of blood cell levels and (5) removal of worn-out cells are needed.

One of the most deterministic aspects in hematopoietic regulation resides on the differentiation commitment induction of hematopoietic cells so that there is a balance between the degree of self-renewal and differentiation. Without sufficient levels of proliferation, there will be insufficient number of cells to carry out normal functions; without appropriate degree of differentiation, however, the functional specification is impossible to maintain. Differentiation commitment ensures a coupled proliferation. As a result, an increasing degree of expression of differentiated function is associated with a decreasing proliferative capacity of individual cells. A disruption of this fine balance of proliferative and differentiative programme may finally lead to disorders from lymphoproliferative or myeloproliferative phenotype, myelodysplasia to full-blown leukemia.

1.1.4 Interrupted hematopoiesis -- Leukemia

Leukemia as uncoupled differentiation/proliferation process

Leukemia can be viewed as a population of hematopoietic cells that has progressed to a certain distance in the maturation pathway but the processes of proliferation and differentiation have become uncoupled. The population, usually arise from a single cell, divide rapidly and extensively without completing their differentiation programme. Such diversion from the normal differentiation programme can occur at various stages between the stem cell and the fully differentiated state.

Many of the leukemia arise from the stem cell population composed of the most malignant cells in poorly differentiated states (Sawyers *et al.,* 1991). For instance, acute undifferentiated leukemia, with neither lymphoid nor myeloid lineage markers, probably result from a primitive stem cell. Acute biphenotypic leukemia, with markers of both lineages, may develop from a more committed bipotent cell. More commonly known leukemia are classified according to the predominant hematopoietic lineage manifested and their form of development (acute or chronic). Regarding myeloid lineage, acute myeloid (myeloblastic) leukemia (AML) probably arise from somewhere between pluripotent stem cell and myeloid progenitor, committed in varying degrees to erythroid, granulocytic-monocytic, and megakaryocytic lines. Chronic myeloid (myelogenous) leukemia (CML), on the other hand, probably derive from a stem cell capable of entering both the myeloid or lymphoid lineages, followed by an expansion of the myeloid compartment at the early onset of the disease.

While acute myeloid leukemia involve both an increase in circulating blood cell number and defects in their normal maturation and function at the onset, the uncoupling of differentiation and proliferation is more gradual in CML. During the chronic phase CML, the myeloid progenitor and mature myeloid cell number increases with cell differentiation remained normal. At blast crisis CML, however,

differentiation is blocked at the progenitor stage as well. Other non-leukemic disorders also reveal disorders of either proliferation or differentiation but not both. Myeloproliferative syndrome represents abnormal growth expansion while myelodysplasia is blocked at differentiation. Both of these syndromes can finally develop into AML (Sawyers *et al.,* 1991). As these phenomena suggest, although we seldom study differentiation without taking into consideration proliferation in normal hematopoiesis, the two processes appear to be independently controlled. Also, the malignant leukemia development seem to require abnormalities in both processes, or in other words, a defect in differentiation commitment.

Leukemia as signaling and genetic defects

Leukemia is a heterogeneous group of diseases caused by accumulation of multiple genetic alternations. These changes can occur in multiple steps and in various sequences of order. They are also influenced by both genetically-determined and environmental factors. Activation of oncogenes which signal proliferation in cells or inactivation of anti-oncogenes (tumor suppressor genes) which slow down cell growth and/or enhance cell differentiation may occur, in either the signal transduction pathway or the gene regulation program. Modulations in signal transduction are exemplified by oncogenes coding for growth factors (*sis,* platelet-derived growth factor), growth factor receptors (*fms,* M-CSF receptor; *kit,* SCF receptor) and intra-cellular signal transducers (*ras,* G protein, *raf,* serine-threonine kinase, *src* family, cytoplasmic tyrosine kinases). On the other hand, changes in gene regulation programme are found in nuclear oncogenes encoding transcription factors (*jun, fos, myc, myb, ets*), nuclear tyrosine kinase (*abl*), hormone receptor (*erb-A*) along with tumor suppressor genes producing transcriptional activator (IRF-1, interferon-regulatory factor-1) and nuclear phosphoproteins (*p53, Rb*) (Clemens, 1991; Hoffbrand and Pettit, 1993; Pallister, 1994). The altered genes themselves may deviate from the normal structures, express at an improper level or in an inappropriate time. In general, transformation of hematopoietic cells needs at least two mutations. For example, over-expression of either IL-3 or HOX B8 (HOX 2.4) gene was not highly leukemogenic (but myeloproliferative in the case of IL-3) in normal bone

marrow cells. Cells enforced to express both genes result in myeloid leukemia *in vitro* and *in vivo* (Perkins *et al.,* 1990). Other two-step combinations or co-operations in myeloid leukemia include *myc/raf, myc/ras, myc*/M-CSF (Adams and Cory, 1992; Anfossi *et al.,* 1989) and *hox-a/Meis1* (Nakamura *et al.,* 1996).

1.2 Regulation of myeloid cell differentiation

1.2.1 Regulation of hematopoiesis

Given that both inappropriate signaling and/or deregulated gene expression result in leukemia, knowledge on normal hematopoiesis regulation, in particular, differentiation commitment may be useful to restore normal blood cell formation.

As a general rule, regulation is effected by both positive signals that stimulate hematopoiesis and negative control system which checks excessive production or fluctuations after amplified response. Control of hematopoiesis is mediated by various hematopoietins or growth factors. In the microenvironment of bone marrow, stromal cells produce soluble factors which act on stem/progenitor cells by paracrine stimulation or inhibition (Verfaillie, 1993). Alternatively, they can effect their actions through membrane-bound growth factors in a cell-cell contact manner (Toksoz *et al.*, 1992). This localized control prevents exhaustive proliferation of stem cells, triggers early differentiation and suppresses terminal differentiation of cells at primitive stages. More dramatic and rapid response of the system can be mediated by humoral growth factors during acute infections and immune response. These growth factors, produced by detector or immune cells in the circulation, stimulate generation of mature cells for body defence. For instance, GM-CSF, G-CSF, M-CSF, IL-1 and IL-6 are produced by macrophages while IL-3, GM-CSF and IL-5 are produced by T cells. Humoral growth factors are also responsible for the final stage differentiation and maturation of circulating blood cells.

Various colony-stimulating factor (CSF), interleukin (IL), erythropoietin (Epo), stem cell factor (SCF), leukemia inhibitory factor (LIF), transforming growth factor-beta (TGF-β), macrophage inflammatory protein-alpha (MIP-1α) and tumor necrosis factor-alpha (TNF-α) are regulators of hematopoiesis. They are very potent acting at nano- to pico-molar concentrations. These growth factors can be stimulatory or inhibitory acting on different lineages (IL-4) or different stages (G-CSF) of cells.

On the other hand, the actions of some factors are restricted to certain lineage (M-CSF, G-CSF, Epo) or stage (IL-3) of cells. In addition, these factors can act in a synergistic manner in triggering hematopoietic cell production. Generally speaking, early-acting factors in the stimulation of dormant stem cell cycling and differentiation consist of IL-6, G-CSF, IL-11, IL-12, LIF, SCF and *flt3/flk-2* ligand. They can act synergistically with one another (SCF + IL-11) or with other intermediate-acting factors (IL-3, IL-4). Intermediate-acting factors such as IL-3, GM-CSF and IL-4 have overlapping functions to ensure the development of multi-potential progenitors. Late-acting factors such as Epo, M-CSF, G-CSF and IL-5 mediate proliferation and maturation of committed progenitors. In contrast, lineage non-specific inhibitory factors IFN-γ and TNF-α, together with lineage specific inhibitory factors TGF-β (acts predominantly on primitive hematopoietic cells and lymphoid cells) and MIP-1α (acts on primitive myelopoietic cells) represent a negative system to control blood cell generation (reviewed in Metcalf, 1988; Metcalf and Nicola, 1995, Ogawa, 1993)

Although regulatory role of hematopoiesis has been imposed on growth factors, the mechanism by which they act remain largely unknown. These growth factors may have acted by changing the gene expression of the cells, producing products necessary for growth and/or differentiation of the cells. Several models have been established trying to explain the control of hematopoiesis in terms of both differentiation commitment and lineage commitment.

1.2.2 Models of hematopoiesis

Inductive model

The inductive model proposed that receptor bindings of the hematopoietic growth factors, perhaps in combination with other components such as cell adhesion molecules and the extracellular matrix of the cellular microenvironment (hematopoietic inductive microenvironment, HIM), induce the differentiation and determine lineage choice from multi-potent cells.

A couple of evidence indicated that differentiation and lineage commitment may have occurred under the control of growth factors in the environment (reviewed in Metcalf and Nicola, 1995).

1. Mouse stem cells developed into different cell lineages according to where they were injected. The cells developed into T cells when they were injected intra-thymically but committed into various lineages when they were injected intra-venously (Spangrude *et al.,* 1988).

2. Stem cells developed into different lineages according to different combinations of growth factors added *in vitro* (Heimfeld *et al.,* 1991; Metcalf, 1991; Metcalf, 1993).

3. Granulocyte-macrophage progenitor cells (Metcalf and Burgess, 1982) and certain multi-potential cell lines (Heyworth *et al.,* 1990) developed into different lineages influenced by the colony-stimulating factors (CSFs).

Stochastic model

The stochastic model explained that stem cells express the genetic changes required for various events in hematopoiesis without exposing to extrinsic stimuli.

Growth factors simply permit the proliferation, development and maturation of these intrinsically committed cells.

A number of observations suggested random processes have occurred in both the determination to differentiate (or to self-renew) and lineage specification during hematopoiesis (reviewed in Ogawa 1993).

The decision of stem cells to either self-renew or to differentiate is a random event as suggested by the following evidence.

1. The distribution of CFU-S formed from individual spleen colonies met the prediction of a stochastically-based birth and death model (Till *et al.,* 1964).

2. The same conclusion as in (1) resulted from analyzing secondary colonies formed by macroscopic erythroid colonies (Humphries *et al.,* 1981) and individual blast cell colonies (Nakahata *et al.,* 1982).

Differentiation and lineage commitment may also be a random process according to the following findings.

1. Multi-lineage colonies of single cell origin or from paired progenitors showed a variety of lineage combinations and a variation in lineage numbers (Suda *et al.,* 1983; Suda *et al.,* 1984).

2. The relative proportions of granulocyte-macrophage, erythroid and multi-potent progenitors remained similar irrespective of the added cytokine combinations (Mayani *et al.*, 1993).

3. Differentiation occurred independent of both proliferation and action of growth factors; growth factors were suggested to have served as survival factors (Fairbairn *et al.,* 1993).

Hybrid model

The major divergence between the inductive and stochastic models lies on the role of growth factors. Whereas the inductive model described growth factors as deterministic, the stochastic model considered it play a minor role. Given the conflict between the above models, the hybrid model considered aspects of both models. Growth factors alter the balance between proliferation and differentiation with differentiation lineage determined by the cells in a stochastic manner. This argument was based on the observation that at least two signaling pathways initiated by two different activated growth factor receptors were required to stimulate differentiation. While differentiation was blocked by one of the pathways, the other did not determine the lineage restriction (Just *et al.*, 1991).

Perhaps even the determination of stem cells to either self-renew or to differentiate is not an entirely random process. The random parameter, probability for self-regeneration is changeable after the pre-existing stem cell population is artificially destroyed (Metcalf, 1988).

1.2.3 Gene regulation of myeloid cell differentiation and its study

Although no consensus can be reached on how the various events of hematopoiesis are initiated or signaled (by induction or by intrinsically-determined events, Figure 1.4), it is still beneficial and important to know the genetic mechanism underlying hematopoiesis. In particular, identifying master genes that determine differentiation commitment would finally find application in correcting disorders like leukemia.

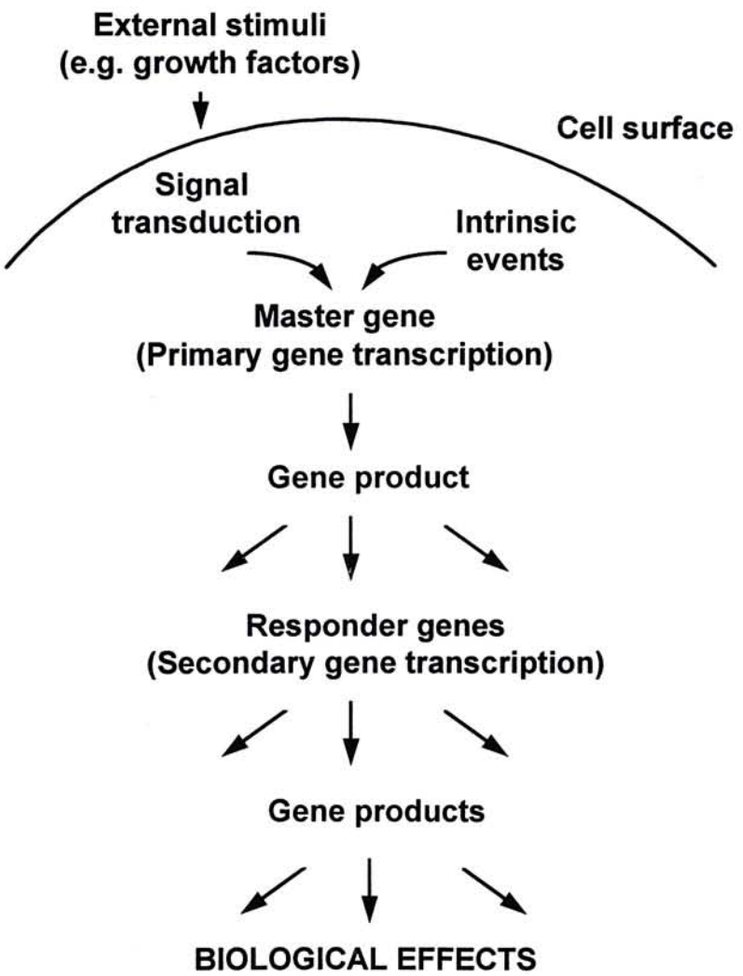


Figure 1.4. **A simplified diagram of signal transduction and cascades of gene regulation.** The genetic program of differentiation may be triggered by external stimuli (inductive model) or intrinsically-determined events (stochastic model). Master gene(s) is/are the target of these signals/events resulting in the modulation of primary gene transcription. The increase or decrease in the master gene products (transcription factors) further initiates an array of changes in secondary gene transcription. The changing level of the responder gene products can then mediate the biological effects required for differentiation. (Clemens, 1991; Rabbitts, 1991)

Master genes refer to genes that control the expression of a set of responder genes (Figure 1.4). They are particularly important as they are at the top of the regulatory cascades, hence having maximum influence over differentiation. They are activated or suppressed (primary gene transcription) by either external stimuli or some yet undefined random events. The gene product of a master gene can be a positive or a negative transcriptional regulator which acts on the secondary transcription events of its responder genes. The gene products of the responder genes can then mediate their biological function necessary for differentiation (Figure 1.4). It is also worth noting that master genes may influence one another through dimerization or competition for binding to the same DNA sequences (Rabbitts, 1991). Changing the expression level of a particular master gene thus affects not only its own set of responder genes but also master genes that have interaction with it. This results in further cascades of change in gene activities. Therefore, 'switching on or off' critical master genes may be able to control the differentiation. Genes that are downstream to these master genes (responder genes) may also be important to differentiation commitment. For example, responder genes that code for growth factor receptors would allow further modulation of the cells by external stimuli relevant to differentiation.

To study the genetic mechanism of differentiation commitment, one of the possible approach is to examine the overall transcriptional activities (or gene expressions) in the cells after they are given a differentiating signal (either extrinsic or intrinsic). That is, to investigate which genes are being switched on or off during the differentiation. However, change in transcriptional activity (or mRNA level) of a gene represents only a correlation relationship of the gene with the differentiation process. It is expected that a lot of genes that are peripheral to in addition to those that are central to differentiation commitment would be isolated. The reason is that genes that are responsible for growth arrest, apoptosis as well as cell maturation and function would also be activated or suppressed during the differentiation process. Therefore, we should take further steps to identify genes that direct the differentiation commitment itself.

To prove whether certain genes are responsible for differentiation commitment, there are several direct strategies. Examination of some congenital mutations reveal the roles of some genes. Examples can be found in M-CSF (*op* mutation), stem cell factor (*Steel, Sl* mutation) and stem cell factor receptor (c-*kit, W* mutant). For genes where such mutations are not found, ectopic expression of the genes or inhibited expression of the genes by either anti-sense, antibody or dominant negative mutant approach can be used. These strategies interfere the endogenous expression of the genes and therefore give us a clearer idea of the gene functions. Alternatively, germ line mutation of the genes in animals can be established and any genes that are in suspicion of a role in cell differentiation commitment can be inspected.

1.2.4 Genes differentially expressed and involved in myeloid cell differentiation

It has been noted that a significant proportion of leukemia has been classified as myeloid leukemia (Hoffbrand and Pettit, 1993). Thus, to understand differentiation commitment as a whole, the myeloid cell model sounds attractive to begin with. More specifically, we are concerned with how the myeloid progenitors give rise to macrophages and/or granulocytes. A vast number of genes have shown to be differentially expressed during myeloid cell differentiation. Some of these genes already have their roles in myeloid differentiation and lineage commitment be established while others still await for further investigation. Genes that are being regulated during an observed differentiation may code for growth factors, receptors, signal molecules, transcription factors, heat shock proteins and integrins. The following is a brief review stressing on genes that have shown differential expression during myeloid cell differentiation (in normal and/or leukemia cells) and the changes in expression have also been proved to be central to differentiation. These genes can be classified according to their gene products into four groups : cytokines, transcription factors, cyclin-dependent kinase inhibitors and proteins of unknown functions. (The characteristics of those myeloid cell lines involved in the studies described below are depicted in Appendix A2.)

Transcription factors

Transcription factors may be the main 'switches' for myeloid differentiation programme. Many of them are specifically induced or suppressed during myeloid cell differentiation. Their pivotal roles in myeloid cell differentiation were also verified by various gene function studies. Several DNA-binding motifs have been identified in transcription factors (reviewed in Locker, 1996) and these motifs have been used to categorize DNA binding proteins into families. In the followings, the family *myb, myc* (helix-loop-helix leucine zipper), basic helix-loop-helix, zinc finger, ETS domain and homeobox-domain will be discussed.

The MYB Family -- c-*myb*

The *myb* family codes for a unique type of transcriptional activators and comprises c-*myb,* B-*myb,* A-*myb* and the transforming gene v-*myc.* Cellular genes c-*myb,* B-*myb* and A-*myb* contain three functional domains, a DNA-binding domain, a transactivation domain and a negative regulatory domain. v-*myb,* however, is truncated at both the N- and C-terminals.

c-*myb* is a cellular counterpart of v-*myb*, an oncogene of avian retrovirus Avian myeloblastosis virus (AMV) and E26. Mutation of c-*myb* by protein truncation and deregulation of transcription cause myelogenous disease (Shen-Ong *et al.,* 1986) and transformation of hematopoietic cells (Gonda *et al.,* 1996) respectively. Its primary role in myeloid cell differentiation and leukemia was further supported by its expression pattern. Its abundant expression level was down-regulated upon terminal differentiation of normal myeloid precursors (Liebermann and Hoffman-Liebermann, 1989; Sheiness and Gardinier, 1984). Similarly, during the monocytic differentiation of WEHI-3B, M1 and HL-60 cells (Liebermann and Hoffman-Liebermann, 1989; Magrinat *et al.,* 1992) as well as granulocytic differentiation of 32Dc13 cells, its expression level was down-regulated significantly (Wolff *et al.,* 1996).

Further evidence revealed that down-regulation of c-*myb* is specifically essential for the late differentiation of both monocytic and granulocytic lineages. Continuous expression of c-*myb* disrupted the monocytic differentiation of M1 cells at a very early myeloblast stage. The cells developed only partially into macrophages (Selvakumaran *et al.,* 1992; Wolff *et al.,* 1996). Constitutive expression of the gene induced differentiation of WEHI-3B (D^+) cells to the promonocyte stages but not differentiation from promonocytes to monocytes (Yanagisawa *et al.,* 1991). Similarly, over-expression of the gene in 32Dc13 cells did not influence the early stages of granulocytic differentiation (promyelocyte to myelocyte) but inhibited the late stages of differentiation (metamyelocyte and neutrophils) (Wolff *et al.,* 1996). Thus, down-regulation of the gene is essential for terminal differentiation of both monocytic and granulocytic differentiation.

Inhibition of the c-*myb* expression by anti-sense technique in bone marrow cells revealed that the expression of c-*myb* is required for erythroid and myeloid colony formation (Gewirtz and Calabretta, 1988). Severe impairment of erythropoiesis and myelopoiesis was also found in homozygous c-*myb* deficient mice (Mucenski *et al.,* 1991). The knock-out mice were defective in fetal liver hematopoiesis and the subsequent adult-type hematopoiesis. They have reduced number of myeloid progenitors (and other progenitors) (Lin *et al.,* 1996) and died at E15.5 day. In conclusion, c-*myb* expression is required for myelopoiesis and myeloid colony formation and the decreased expression of the gene is essential for terminal differentiation of both monocytic and granulocytic differentiation.

c-*myb* represents one of the master genes of myeloid differentiation. Its responder genes included genes that are responsible for controlling both proliferation and differentiation. Some of them are mainly involved in growth control (e.g. *cdc* 2) while others are differentiation-related (e.g. *mim-1*). Putative downstream genes of c-*myb* in myeloid differentiation include CD34 gene, c-*myb* (auto-regulation), c-*myc, hsp70,* DNA polymerase α gene and *mim-1* (reviewed in Hesketh, 1995). Among these responder genes, CD34 and c-*myc* have been shown to be down-regulated during the myeloid cell differentiation and the decreased expression has also found to be essential to the differentiation (see below).

The MYC family

The *myc* family comprise c-*myc, max (myn,* murine homolog), *mad* and *mxi1*. All the MYC family proteins contain a conserved structure, the basic region helix-loop-helix leucine zipper (bHLH-Zip) which allows DNA binding and oligomerization with other proteins. MYC, in addition, has a transactivation domain in its N-terminal. Whereas MYC does not form homo-dimers or hetero-dimers with either MXI1 or MAD, MAX forms homo-dimers as well as hetero-dimers with all the other three members. In addition, all these dimers have shown to bind on the same E-box sequence (reviewed in Locker, 1996).

c-*myc,* the best known member, was discovered as the cellular homolog of a transforming oncogene in a group of avian retroviruses. It is suggested to be a key regulator in mammalian cell proliferation, differentiation and apoptosis. Its expression level has been shown to decrease upon monocytic differentiation of normal mouse myeloblast (Liebermann and Hoffman-Liebermann, 1989) and various leukemia cell lines (e.g. M1, HL-60, ML-1, K562 and U937 cells) or upon the granulocytic differentiation of HL-60 cells (Hoffman-Liebermann and Liebermann, 1991; Larsson *et al.,* 1994). This decrease in c-*myc* expression level is both essential for myeloid cell differentiation and sufficient to trigger differentiation. Over-expression of c-*myc* was able to block the induced differentiation of several myeloid cell lines like murine J774, M1 and human U937 cells (Chisholm *et al.,* 1992; Hoffman *et al.,* 1996) whereas inhibition of c-*myc* expression by anti-sense oligomer alone was able to cause the differentiation of HL-60 (Holt *et al.,* 1988).

max, was identified by screening a cDNA expression library with the C-terminus of human MYC. Its expression was decreased during the monocytic differentiation of normal myeloblasts (Nguyen *et al.,* 1995), HL-60, M1, ML-1 and U937 cells, as well as during the granulocytic differentiation of HL-60 cells (Larsson *et al.,* 1994). The action of *max* on myeloid cell differentiation appeared to be tightly linked to other family members. Blocking expression of either c-*myc* or *max* by anti-sense oligomers in M1 cells inhibited proliferation and induced differentiation of the cells to the intermediate stage. Terminal differentiation was only observed when both genes were blocked in M1 cells. On the other hand, inhibition of either c-*myc* or *max* by anti-sense oligomer was sufficient to suppress growth and accelerate the differentiation of GM-CSF-stimulated myeloblast enriched BM cells along both the monocytic and granulocytic lineages. However, combined actions of both oligomers did not enhance the effect on differentiation (Nguyen *et al.,* 1995). Taken together, decreased expression of either c-*myc* or *max* enhances the differentiation of bone marrow cells. In addition, down-regulation of both c-*myc* and *max* is essential for terminal differentiation of myeloid leukemia cells.

mad is isolated as a result of screening a λgt11 expression library using MAX as probe (Ayer *et al.,* 1993). The gene expression was up-regulated in various myeloid cell lines during their induced differentiation into macrophages (e.g. HL-60, K562, ML-1 and U937 cells) or granulocytes (e.g. HL-60) (Larsson *et al.,* 1994). In normal myeloblasts and monoblasts, rapid induction of *mad* was also observed upon differentiation (Hurlin *et al.,* 1994). Nevertheless, the exact role played by the gene has not been demonstrated.

mxi1 was found as MXI1 specifically interacts with MAX (Zervos *et al.,* 1993). Its expression has shown to be increased and remained elevated at all times during monocytic differentiation of U937 (Larsson *et al.,* 1994; Zervos *et al.,* 1993). In contrast, *mxi1* expression was strongly reduced in the monocytic differentiation of K562 (Delgado *et al.,* 1995). These different patterns of expression may suggest different action of the genes in cells at different differentiation stages but such view has not been established by solid evidence.

The expression pattern of the *myc* family also suggested some degree of lineage specificity. While c-*myc* expression was consistently decreased during both the monocytic and erythroid differentiation of K562 cells, the expression level of *max* was down-regulated during erythroid differentiation but unchanged in expression during monocytic differentiation of K562 cells. *mxi1* was down-regulated during the monocytic differentiation of K562 cells but up-regulated during their erythroid differentiation. In contrast, the expression of *mad* was elevated during the monocytic differentiation of K562 cells but remained unchanged during its erythroid differentiation (Delgado *et al.,* 1995). These expression patterns suggested the members of the *myc* family are regulated differently during different lineage specification. However, whether a decrease in expression of c-*myc* and *mxi1,* an unchanged mRNA level of *max* and an increase in *mad* expression are causative to myelomonocytic in preference to erythroid differentiation are unknown.

Some potential target genes of c-*myc* such as ornithine decarboxylase (ODC) have been identified. However, none of them have been proved to be essential for myeloid cell differentiation (reviewed in Hesketh, 1995; Hoffman *et al.,* 1996).

Basic helix loop helix -- SCL

SCL gene, codes for a basic helix-loop-helix (bHLH) protein, was cloned from t(1;14) translocation of a patient with biphenotypic leukemia. Recently, deregulation of SCL gene was also suggested in myeloid leukemia cells (Tanigawa *et al.,* 1994). During myeloid cell differentiation of K562 cells, there was an early transient fall in SCL mRNA levels. This level was then increased but dropped again at a later time (Green *et al.,* 1993). In the induced differentiation of FDCPmix A4 cells to the granulocyte/monocyte lineage, a marked decrease in SCL mRNA was also observed (Cross *et al.,* 1994). Similarly, a late and persistent decrease in mRNA levels was found during the monocytic differentiation of M1 cells (Begley, 1994). In contrast, the mRNA level of SCL gene was decreased only transiently, increased later and maintained throughout the erythroid differentiation of erythroleukemia MEL (Green *et al.,* 1993). Up-regulation of SCL gene was also found during the erythroid differentiation of FDCPmix cells (Cross *et al.,* 1994). Constitutive expression of SCL gene inhibited LIF- and OSM- induced differentiation of M1 cells, indicating that down-regulation of SCL gene is specific for and essential to myeloid cell differentiation (Begley 1994; Tanigawa *et al.,* 1993).

Leucine zipper -- CCAAT / Enhancer Binding Protein -- C/EBP

C/EBP is a family of basic leucine zipper binding proteins which bind DNA as homo- or hetero-dimers. The members of the family include C/EBPα, C/EBPβ (NF-IL6, LAP, IL-6DBP, AGP/EBP and CRP2), C/EBPγ (Ig/EBP-1) and C/EBPδ (CRP3). C/EBPα, C/EBPβ and C/EBPδ mRNAs were detected and appeared to be restricted to myeloid cells (Natsuka *et al.,* 1992). Differentiation-related changes of the gene

expression further suggest their roles in myeloid cell differentiation. High level of C/EBPα expression was detected in myelomonocytic cells but the level was down-regulated on maturation. Its expression was also decreased during monocytic differentiation of HL-60 cells and granulocytic differentiation of both HL-60 and 32Dcl3 cells (Scott *et al.,* 1992). C/EBPβ (NF-IL6), in contrast, was increased in mRNA level during the monocytic differentiation of HL-60, M1 and U937 cells and during granulocytic differentiation of 32Dc13 cells (Natsuka *et al.,* 1992). Conversely, no change in expression level was accompanied with the granulocytic differentiation of HL-60 (Natsuka *et al.,* 1992). For C/EBPδ, the expression was up-regulated during the granulocytic differentiation of 32Dcl3 (Scott *et al.,* 1992).

C/EBP family also regulate certain myeloid-related or specific genes. C/EBPβ has been shown to regulate IL-1β and TNF-α (Pope *et al.,* 1994; Tsukada *et al.,* 1994). On the other hand, C/EBP binding sites have been located in the proximal promoter region of G-CSFR, GM-CSFRα and M-CSFR where PU.1 sites are also found (Zhang *et al.,* 1996). Recent studies have pointed out that C/EBPα would be the major form that binds and activates such promoters in myeloid cell lines (Hohaus *et al.,* 1995; Zhang *et al.,* 1994). Regulation of GM-CSFRα by C/EBPα plus PU.1 have been demonstrated in monocytic cells (Hohaus *et al.,* 1995). The regulation of these receptor genes by C/EBPα (and other transcription factors) would probably alter the response of the myeloid cells to exogenous growth factor. However, it is also worth noting that other family members in addition to C/EBPα also target on this promoter site. In addition, homo- or hetero-dimers between the family members may form (Scott *et al.,* 1992; Zhang *et al.,* 1996). As a result, the relative amount of these members during myeloid differentiation may affect the expression of their target genes through binding competition and/or dimerization. Further studies on these genes may reveal a complex system of myeloid cell differentiation.

Leucine zipper -- The FOS/JUN family

The *fos/jun* family (c-*fos*, *fosB*, *fra1*, *fra2*, c-*jun*, c-*jun*B, c-*jun*D) code for ubiquitous leucine zipper transcription factors. c-*jun* and c-*fos* are two prominent members. They are readily induced as primary response genes and activated in response to growth factors and agents like phorbol esters (e.g. PMA). Also, c-*jun* can form homo-dimers or hetero-dimers with c-*fos* to become activator protein-1 (AP-1).

The possible role(s) of the *fos/jun* genes and AP-1 complex in myeloid cell differentiation can largely be reflected in their expression patterns in myeloid cells. The expression levels of c-*fos* and c-*jun* were readily increased during the leukemia cell differentiation of U937, HL-60 and some leukemic cell clones (Adunyah *et al.*, 1992; Hass *et al.*, 1991; Shabo *et al.*, 1990; Szabo *et al.*, 1991). Besides, other family members like *jun*B and/or *jun*D (having sequence homology with c-*jun*) were enhanced within hours in mRNA levels during the induced monocytic differentiation of HL-60, M1, THP-1 and U937 cells (Datta *et al.*, 1991; Lord *et al.*, 1990a; Mollinedo and Naranjo, 1991) or granulocytic differentiation of HL-60 cells (Mollinedo and Naranjo, 1991).

The roles of *fos/jun* in myeloid cell differentiation were further supported by more direct evidence. Enforced expression of c-*jun* was capable of initiating differentiation of WEHI-3B (D^+) cells (Li *et al.*, 1994c) and partial differentiation of U937 cells (Szabo *et al.*, 1994) in the absence of differentiating agent. In addition, enforced expression of c-*fos* increased the propensity of M1 cells to differentiate and reduced their leukemic phenotype. c-*fos* anti-sense oligomer, on the other hand, was able to block *in vitro* terminal differentiation of normal myeloblasts significantly (Lord *et al.*, 1993). Thus, c-*jun* alone is sufficient to trigger monocytic differentiation of leukemia cell lines while c-*fos* is essential for differentiation of normal myeloblasts and myeloid leukemia cells.

c-*jun* and AP-1 binding may also determine the lineage choice of myeloid cells and progenitor cells. mRNA level of c-*jun* was increased during the induced

monocytic differentiation of U937 and HL-60 cells but not granulocytic differentiation of HL-60 cells (Mollinedo and Naranjo 1991; Gaynor *et al.,* 1991). AP-1 binding activity was increased during the monocytic differentiation of HL-60 cells but not granulocytic differentiation of HL-60 cells (Mollinedo *et al.,* 1993). Hence, it may be rewarding to conduct direct strategies like anti-sense technique or ectopic expression strategies (in an appropriate system) to see if the gene is deterministic in lineage commitment.

Zinc finger -- EGR-1

egr-1, encodes a zinc finger transcription factor (EGR-1, Krox24, NGIF-A, Zif/268, Tis8), was first identified in 3T3 fibroblast-like cells. It is expressed during the differentiation of nerve, bone and myeloid cells and during the maturation of B cells. It is a primary response gene induced specifically during monocytic but not granulocytic differentiation. It is transiently expressed upon monocytic differentiation of HL-60, M1 and U937 cells (Nguyen *et al.,* 1993). However, no increase in expression was found during the granulocytic differentiation of HL-60 cells (Nguyen *et al.,* 1993). Similarly, the mRNA level of *egr-1* was increased in normal myeloblast induced to follow the monocytic pathway but not in bone marrow cells induced to follow the granulocytic pathway (Nguyen *et al.,* 1993). *egr-1* is essential for monocytic but not granulocytic differentiation. Inhibition of the gene expression by anti-sense oligomer blocked the monocytic differentiation of HL-60, U937, M1 and myeloblast-enriched bone marrow cells but not granulocytic differentiation of HL-60 cells (Nguyen *et al.,* 1993). Enforced expression of the gene prevented HL-60 cells from moving along the granulocytic but not the monocytic lineage (Nguyen *et al.,* 1993). Taken together, increased expression of *egr-1* is essential specifically in monocytic differentiation. Moreover, enforced expression of the gene can override the granulocytic differentiation program of the cells.

One potential target gene of EGR-1 is c-*myb* (another important gene in myeloid cell differentiation, see above). c-*myb* is expressed when granulocytic

differentiation of HL-60 was blocked by enforced expression of e*gr-1*. Having at least one EGR-1 binding site on its promoter (Nicolaides *et al.,* 1991), c-*myb* is suggested to be transactivated by EGR-1 and prevent the granulocytic differentiation (Nguyen *et al.,* 1993).

Zinc finger -- Myeloid Zinc Finger MZF-1

While *egr-1* is important for monocytic differentiation, another zinc finger gene *mzf-1* is essential to granulopoiesis.

mzf-1 was originally probed from a myeloid λgt11 cDNA library made from a patient with CML, using conserved sequence of the zinc finger genes. (Hromas *et al.,* 1991). In fact, *mzf-1* has been proved to be a typical zinc finger gene. Its zinc finger domain is also related to PLZF-1, part of a fusion protein with RAR-α, in t(11;17) promyelocytic leukemia (Chen *et al.,* 1993).

The *mzf-1* mRNA could be detected in various leukemia cell line including HL-60, KG-1, HEL and K562 (Hromas *et al.,* 1991). The gene is also expressed exclusively in myeloid cells in normal bone marrow, from myeloblasts to metamyelocytes (Bavisotto *et al.,* 1991). Moreover, during the induced granulocytic differentiation of HL-60 cells, the gene increased in expression (Hromas *et al.,* 1991). This increase in expression was later proved to be essential in granulopoiesis *in vitro.* Inhibition of *mzf-1* by anti-sense oligomer decreased the formation of granulocyte colonies (G-CFU) but not erythroid colonies (BFU-E). Also, the formed granulocytic colonies were dysplastic in morphology (Bavisotto *et al.,* 1991). Continuous over-expression of *mzf-1* in the FDCP.1 myeloid cell line, on the other hand, prevented apoptosis and altered the phenotype of the cells which caused tumor formation in mice (Hromas *et al.,* 1996). These findings suggested the gene is crucial to myeloid colony formation and that deregulated expression of the gene causes transformation.

ETS family -- PU.1

PU.1 is a member of the ETS transcription factor family. PU.1 gene is expressed exclusively in the hematopoietic system, highly expressed in granulocytic, monocytic and B lymphocytic cells. It is positively auto-regulated in myeloid cells but not in T cells nor in non-hematopoietic cells (Chen *et al.,* 1995). PU.1 has been suggested to be a key regulator in myelopoiesis at both early and more mature stages. PU.1 is expressed in the earliest multi-potential progenitors and specifically up-regulated during commitment to the myeloid lineage (Voso *et al.,* 1994). Another report revealed that the gene was expressed in early myeloid precursors but declined in expression as the cells differentiated towards the granulocytic pathway (Hromas *et al.,* 1993a). Inhibition of PU.1 function by competitor oligonucleotides blocked myeloid colony formation in human $CD34^{+}$ cells (Voso *et al.,* 1994). Also, inactivation of the gene by homologous recombination showed a multi-lineage defect in mice suggesting that PU.1 may have affected myeloid cell differentiation at the early multi-potential progenitor stage (Scott *et al.,* 1994). Recent studies on examination of embryonic and fetal hematopoietic organ from $PU.1^{-/-}$ mice and *in vitro* differentiation of $PU.1^{-/-}$ ES cells, however, showed that PU.1 had a notable effect on late (CD11b, M-CSFR and CD64) rather than early myeloid gene expression. This suggested PU.1 may have an important role in later development of myeloid cells as well (Simon *et al.,* 1996).

Homeobox-containing genes

The homeobox is a DNA region of 180/183 base pair long encoding a conserved 60/61 amino acid domain, the homeodomain. It has a structure very similar to the helix-turn-helix motif (Billeter *et al.,* 1990). Homeobox genes can be divided into different classes, superclasses and families based on the structural similarities and evolutionary relationship (reviewed in Burglin, 1994). For simplicity, homeobox-containing genes can be roughly divided into two main groups : the HOX clusters and the non-cluster members. Homeobox-containing genes have been well-known for

their roles in embryogenesis and now there is increasing evidence that these genes are also important in hematopoietic cell differentiation (Giampaolo *et al.,* 1995; Lawrence and Largman, 1992; Magli *et al.,* 1991; Sauvageau *et al.,* 1994; Shen *et al.,* 1989; Vieille-Grosjean *et al.,* 1992).

The possible roles of individual homeobox genes in myeloid cell differentiation are suggested by the differentiation-related changes of gene expression. The most remarkable example is HOX B7. A rapid expression of HOX B7 was shown during the vitamin D_3-induced monocytic differentiation of HL-60 cells (Lill *et al.,* 1995). In addition, HOX B7 gene expression was detected in human bone marrow cells differentiating into the myeloid lineage (Lill *et al.,* 1995). Over-expression of the gene was able to inhibit the granulocytic differentiation of HL-60 cells while no effect was observed on the monocytic differentiation (Lill *et al.,* 1995). Inhibition of the HOX B7 gene expression by anti-sense oligomers was able to inhibit markedly the formation of colonies derived from GM-CSF-stimulated bone marrow cells. A similar observation had also been noted in murine bone marrow where anti-sense oligomer directed against mRNA of HOX B7 gene was able to block myeloid colony formation without affecting erythroid or megakaryocytic differentiation (Wu *et al.,* 1992). These functional studies have proved that the gene is essential for myeloid colony formation. It can also override the granulocytic differentiation of myeloid cells when over-expressed. However, the gene alone has no effect on monocytic differentiation.

In addition to the HOX cluster, non-cluster homeobox genes may also play a role in myeloid cell differentiation. *Prh* (Proline-rich homeobox) is a novel human homeobox-containing gene isolated from a $CD34^+$ cell line KG-1A. Its expression is mainly restricted to hematopoietic tissue (Hromas *et al.,* 1993b). *Prh* mRNA was found to express in early myelomonocytic and erythromegakaryocytic cell lines. Upon *in vitro* differentiation of the cell line into mature monocyte/macrophage, *Prh* expression was lost. This down-regulation of expression level was also observed in normal monocyte but the gene was highly expressed in normal granulocytes (Manfioletti *et al.,* 1995). *Hlx* (homology of human *HB*24) may also be important in myeloid cell differentiation. Its expression was restricted to myelomonocytic and pre-

B lineage and increased on maturation (Harvey and Adams, 1991). The phenotypic myeloid maturation of FDC-P1 was also associated with the induction of *Hlx* expression (Allen and Adams, 1993). However, the significance of the decrease or increase in expression of *Prh* and *Hlx* in relation to myeloid cell differentiation is not known until further investigation is carried out.

Cytokines

Cytokines, in particular, the hematopoietins are regulators of hematopoiesis. Some of them, together with their receptors, are particularly important in the myeloid lineage. Exogenous effects of these cytokines on myeloid cell differentiation have been vigorously studied. They are also suggested to be external stimuli that drive the differentiation commitment and bias the lineage choice. Interestingly, newly transcribed cytokine genes (IL-1, IL-6 and TNF-α) were also found to be associated with the monocytic differentiation of both normal differentiating bone marrow-derived colonies (Witsell and Schook, 1992) and monocytic leukemia cell lines (Ishizuka *et al.*, 1995; Lotem and Sachs, 1989; Magrinat *et al.*, 1992). One of the most notable examples was TNF-α. The expression level of the gene was increased significantly during the monocytic differentiation of U937 and HL-60 cells as well as PMA- or TNF-α induced differentiation of WEHI-3B JCS cells (Hass *et al.*, 1991; Magrinat *et al.*, 1992; Mak *et al.*, 1993). In addition, its expression was also increased in the differentiating bone marrow-derived macrophage colonies (Witsell and Schook, 1992). Further investigation suggested endogenous TNF-α is essential for monocytic differentiation of both the normal and leukemia cells. The PMA- or TNF-α induced monocytic differentiation of WEHI-3B JCS cells could be abrogated by TNF-α neutralizing antibodies (Mak *et al.*, 1993). Likewise, anti-sense oligomers against TNF-α messages were able to maintain the proliferation state of the growth factor-induced bone marrow-derived macrophages without terminal differentiation (Witshell and Schook, 1992).

Cyclin-dependent kinase inhibitors (Cdk inhibitors) -- p21

$p21^{WAF, CIP1}$, a cyclin-dependent kinase inhibitor, is involved in the blocking of cell cycle progression. Its gene expression was increased during the differentiation of several cell types including the myeloid leukemia cell lines HL-60 and U937 (Jiang *et al.*, 1994; Liu *et al.*, 1996). Unexpectedly, the gene is not only involved in events in the cell cycle but also initiates the myeloid cell differentiation. It was transcriptionally activated during the monocytic differentiation of U937 cells induced by vitamin D_3. In the absence of differentiation inducers, transient over-expression of the p21 gene was able to drive the cells to differentiate into mature stage (Liu *et al.*, 1996). Nevertheless, the gene may not be essential to myeloid cell differentiation as $p21^{-/-}$ mice showed only defects in the ability to arrest in G_1 phase (Deng *et al.*, 1995).

Protein of unknown function-- CD34

CD34 is a well-known and widely-used marker for identifying hematopoietic stem/progenitor cells. However, its function remains unknown. It is thought to be a type I transmembrane protein and highly O-glycosylated on the extracellular region. The ability of CD34 to bind to L-selectin (Baumheuter *et al.*, 1993) suggested that CD34 might play a role in cellular interaction and adhesion in stromal microenvironment. CD34 has an alternative truncated form in addition to the membrane-bound form (Suda *et al.*, 1992). While the full-length CD34 mRNA was detected in all $CD34^+$ cell lines tested, the truncated form was not.

CD34 gene expression was decreased during the monocytic differentiation of M1 cells (Fackler *et al.*, 1995). Recent studies further revealed that CD34 is essential to myeloid cell differentiation. Enforced expression of transmembrane CD34 without cytokine induction could initiate the differentiation of M1 but not HL-60 nor 32D cells to an intermediate stage (Fackler *et al.*, 1995). However, down-regulation of CD34 (transmembrane form) is essential to terminal differentiation of M1 cells. While over-expression of the truncated CD34 did not affect the induced terminal

differentiation of M1 cells, constitutive expression of the full-length form prevented the cells from terminally differentiated (Fackler *et al.,* 1995). Taken together, down-regulation of CD34 (full length) expression alone initiates M1 cell differentiation to an intermediate stage, and its down-regulation is required for mature monocytic differentiation. The important role played by CD34 in myeloid cell differentiation was further confirmed in transgenic studies. First, delayed erythroid and myeloid differentiation was found in yolk sac-like hematopoiesis in embryoid bodies generated from $CD34^{-/-}$ ES cells. Second, the colony-forming activities decreased in both yolk sacs and fetal liver progenitors in $CD34^{-/-}$ embryo; in addition, there was also decreased colony-forming activities of hematopoietic progenitors in adult $CD34^{-/-}$ mice (Cheng *et al.,* 1996). In conclusion, CD34 is essential for progenitor cell formation including that of the myeloid lineage in both adult and embryonic hematopoiesis.

Differentially expressed genes during myeloid cell differentiation -- an overview

A lot of genes have been described to date to be differentially expressed or transcriptionally activated or suppressed in the course of myeloid cell differentiation. In this review, we are concerned about genes that are specifically regulated during the differentiation of the relatively committed precursor cells which have already restricted their differentiation pathways to monocytic or granulocytic pathways. These genes that change in their mRNA levels during differentiation may be cytokines, cytoplasmic tyrosine kinases, cyclin-dependent kinase inhibitors, heat shock proteins, transcription factors or proteins of unknown functions. Among these genes, however, only some of them are relevant to the differentiation commitment. Gene products that have been proved to be primary to differentiation commitment were interestingly limited largely to transcription factors (summarized in Table 1.1). The phenomenon may be attributed to the fact that transcription factor genes are master genes that control the differentiation program at a top level. However, it may not be at all wise to restrict our search only to transcription factors, as responder genes like CD34, Cdk inhibitor and cytokines have also been proved to be either essential or sufficient factors to cause differentiation. Although genes for MyD88 (Lord *et al.,* 1990b) and

heat shock proteins (*hsp27, hsp70*) (Minowada and Welch, 1995) were shown to have marked changes in expression level, their functions during myeloid cell differentiation remain to be determined. Hence, a comprehensive search for all differentially expressed genes during myeloid cell differentiation would be rewarding.

It must also be stressed that both 'switching on' or 'switching off' of genes are important for myeloid cell differentiation program. Both up-regulation (c-*jun,* c-*fos, egr-1, mzf-1,* HOX B7 gene, TNF-α and p21) and down-regulation (c-*myb,* c-*myc, max,* SCL gene, PU.1 and CD34) of genes have proved to be equally crucial in myeloid cell differentiation (Table 1.1). Thus, to obtain a complete picture of myeloid cell differentiation, studies on both type of genes are necessary.

Finally, there are genes that have different actions on two different cell lineages (e.g. *egr-1,* HOX B7 gene). While they are essential for the differentiation of one lineage, they suppress the differentiation of another. As a result, studying genes that are specifically up-regulated in one lineage but not the other may reveal their differential effects on two lineages (e.g. *egr-1*).

In short, we need to employ a searching strategy which aims at finding all differentially regulated genes during myeloid cell differentiation. The system should also allow the identification of up-regulated and down-regulated genes at the same time. A comparison of the expression patterns of these genes during both monocytic and granulocytic differentiation should also be performed.

Table 1.1. **List of genes differentially expressed during myeloid cell differentiation and their proved functions in differentiation.**

Gene	Gene type	Expression pattern during myeloid cell differentiation	Proved function in myeloid cell differentiation / myelopoiesis (technique used)	Interaction with other genes
c-*myb*	Transcription factor, *myb* family	Down-regulation during monocytic differentiation of • HL-60, M1, WEHI-3B(D^+) cells • normal myeloid precursors Down-regulation during granulocytic differentiation of • 32Dcl3 cells (Liebermann and Hoffman-Liebermann, 1989; Magrinat *et al.*, 1992; Sheiness and Gardinier, 1984; Wolff *et al.*, 1996)	Decreased expression essential for late differentiation of • M1, WEHI-3B(D^+) cells to macrophage (Continuous gene expression) • 32Dcl3 cells to granulocyte (Gene over-expression) Essential for myeloid colony formation (Anti-sense technique) Essential to myelopoiesis (Knock-out mice) (Gewirtz and Calabretta, 1988; Liu *et al.*, 1996; Mucenski *et al.*, 1991, Selvakumaran *et al.*, 1992; Wolff *et al.*, 1996; Yanagisawa *et al.*, 1991)	Target genes : c-*myb*, c-*myc*, CD34 gene, *mim-1*, *hsp70* Regulated by *egr-1* (putative) (reviewed in Hesketh, 1995; Nicolaides *et al.*, 1991)
c-*myc*	Transcription factor, *myc* family	Down-regulation during monocytic differentiation of • HL-60, M1, ML-1, K562 and U937 cells • normal myeloblast Down-regulation during granulocytic differentiation of • HL-60 cells (Liebermann and Hoffman-Liebermann, 1989; Larsson *et al.*, 1994)	Decreased expression essential for myeloid cell differentiation of • J774, M1 and U937 cells (Gene over-expression) Decreased expression enough to trigger myeloid cell differentiation of (Anti-sense oligomer) • HL-60 cells (Chisholm *et al.*, 1992; Hoffman *et al.*, 1996; Holt *et al.*, 1988)	Target gene : gene coding for ornithine decarboxylase (ODC) Regulated by : c-*myb* Co-operative gene : *max* (reviewed in Hesketh, 1995; Hoffman *et al.*, 1996; Nauyen *et al.*, 1995)

max	Transcription factor, *myc* family	Down-regulation during monocytic differentiation of • HL-60, M1, ML-1 and U937 cells • normal myeloblasts Down-regulation during granulocytic differentiation of • HL-60 cells (Larsson *et al.*, 1994; Nguyen *et al.*, 1995)	Decreased expression triggered monocytic differentiation (Anti-sense oligomer) • M1 cells • GM-CSF induced myeloblast-enriched bone marrow cells (Nguyen *et al.*, 1995)	Co-operative gene: c-*myc* Putative co-operative genes : *mad, mxi1* (reviewed in Locker., 1996; Nguyen *et al.*, 1995)
mad	Transcription factor, *myc* family	Up-regulation during monocytic differentiation of • HL-60, K562, ML-1 and U937 cells • normal myeloblast and monoblasts Up-regulation during granulocytic differentiation of • HL-60 cells (Hurlin *et al.*, 1994; Larsson *et al.*, 1994)	No direct proof yet	Putative co-operative gene : *max* (reviewed in Locker, 1996)
mxi1	Transcription factor, *myc* family	Up-regulation during monocytic differentiation of U937 cells but down-regulation during monocytic differentiation of K562 cells (Larsson *et al.*, 1994; Zervos *et al.*, 1993)	No direct proof yet	Putative co-operative gene : *max* (reviewed in Locker, 1996)
SCL gene	Transcription factor, basic helix-loop-helix	An overall down-regulation during monocytic differentiation of • K562, M1 and FDCPmix A4 cells (Begley, 1994; Cross *et al.*, 1994; Green *et al.*, 1993)	Down-regulation of expression essential to monocytic differentiation of M1 (Constitutive gene over-expression) (Tanigawa *et al.*, 1993)	

Gene	Gene type	Expression pattern during myeloid cell differentiation	Proved function in myeloid cell differentiation / myelopoiesis (technique used)	Interaction with other genes
C/EBPα	Transcription factor, leucine zipper	Down-regulation during monocytic differentiation of • HL-60 cells Down-regulation during granulocytic differentiation of • HL-60 and 32Dcl3 cells (Natsuka *et al.*, 1992; Scott *et al.*, 1992)	No direct proof yet	Target gene : GM-CSFR Co-operative gene : PU.1 gene (Hohaus *et al.*, 1995)
C/EBPβ	Transcription factor, leucine zipper	Up-regulation during monocytic differentiation of • HL-60, M1 and U937 cells Up-regulation during granulocytic differentiation of • 32Dcl3 cells (Natsuka *et al.*, 1992, Scott *et al.*, 1992)	Up-regulation essential to macrophage function (Target disruption) (Tanaka *et al.*, 1995)	Target genes : IL-1β, TNF-α, *mim-1* Co-operative gene : c-*myb* (Ness *et al.*, 1993; Pope *et al.*, 1994; Tsukada *et al.*, 1994)
C/EBPδ	Transcription factor, leucine zipper	Up-regulation during granulocytic differentiation of 32Dcl3 cells (Scott *et al.*, 1992)	No direct proof yet	
c-*jun*	Transcription factor, leucine zipper	Up-regulation during monocytic differentiation of • U937, HL-60 cells and some leukemic clones (Adunyah *et al.*, 1992; Hass *et al.*, 1991; Shabo *et al.*, 1990; Szabo *et al.*, 1991)	Increased expression able to trigger monocytic differentiation of (Enforced gene expression) • U937 and WEHI-3B(D^+) cells (Li *et al.*, 1994c; Szabo *et al.*, 1994)	

c-*fos*	Transcription factor, leucine zipper	Up-regulation during monocytic differentiation of • U937, HL-60 cells and some leukemic clones (Adunyah *et al.*, 1992; Hass *et al.*, 1991; Shabo *et al.*, 1990; Szabo *et al.*, 1991)	Increased expression essential for monocytic differentiation of (Anti-sense strategy) • normal myeloblasts (Lord *et al.*, 1993)	
*jun*B *jun*D	Transcription factor, leucine zipper	Up-regulation during monocytic differentiation of • U937, HL-60, THP-1, M1 cells Up-regulation during granulocytic differentiation of • HL-60 cells (Datta *et al.*, 1991; Lord *et al.*, 1990a; Mollinedo and Naranjo 1991)	No direct proof yet	
egr-1	Transcription factor, zinc finger	Up-regulation during monocytic differentiation of • HL-60, M1 and U937 cells • normal myeloblasts No induction during granulocytic differentiation of • HL-60 cells • bone marrow cells (Nguyen *et al.*, 1993)	Up-regulated expression essential for monocytic differentiation of (Anti-sense oligomer) • HL-60, U937 and M1 cells • myeloblast-enriched bone marrow cells Enforced expression suppressed granulocytic differentiation of • HL-60 cells (Nguyen *et al.*, 1993)	Putative target gene : c- *myb* (Nicolaides *et al.*, 1991)
mzf-1	Transcription factor, zinc finger	Up-regulation during the differentiation of • HL-60 cells (Hromas *et al.*, 1991)	Essential for the formation of granulocytic colonies (Anti-sense oligomer) Enforced expression caused cell transformation (Bavisotto *et al.*, 1991; Hromas *et al.*, 1991, 1996)	

Gene	Gene type	Expression pattern during myeloid cell differentiation	Proved function in myeloid cell differentiation / myelopoiesis (technique used)	Interaction with other genes
PU.1 gene	Transcription factor, ETS family	Down-regulation during granulocytic differentiation of myeloid precursors (Hromas *et al.*, 1993)	Essential for myeloid colony formation (Competitor oligonucleotides) and myeloid progenitor formation (Targeted mice) (Scott *et al.*, 1994; Voso *et al.*, 1994)	Target gene : GM-CSFRα Putative target genes : M-CSFR, G-CSFR (Hohaus *et al.*, 1995; Zhang *et al.*, 1996)
HOX B7 gene	Transcription factor, homeobox gene	Up-regulation during monocytic differentiation of • vitamin D_3 -induced HL-60 cells (Lill *et al.*, 1995)	Over-expression of the gene suppress granulocytic differentiation of HL-60 cells Essential for myeloid colony formation (Anti-sense technique) (Lill *et al.*, 1995; Wu *et al.*, 1992)	
TNF-α	Cytokine	Up-regulation during monocytic differentiation of • HL-60, U937, WEHI-3B (JCS) cells • Bone marrow-derived macrophage colonies (Hass *et al.*, 1991; Magrinat *et al.*, 1992; Mak *et al.*, 1993; Witsell and Schook, 1992)	Increased expression essential for monocytic differentiation of • PMA- and TNF-induced WEHI-3B (JCS) cells (Neutralizing antibodies) • Bone marrow-derived macrophage colonies (Anti-sense oligomer) (Mak *et al.*, 1993; Witsell and Schook, 1992)	
p21	Cyclin-dependent kinase inhibitor	Up-regulation during monocytic differentiation of • HL-60 and U937 cells (Jiang *et al.*, 1994; Liu *et al.*, 1996)	Transient over-expression sufficient to initiate monocytic differentiation of U937 cells Not essential to myeloid cell differentiation in mice (Target disruption) (Deng *et al.*, 1995; Liu *et al.*, 1996)	Regulated by: Vitamin D (Liu *et al.*, 1996)

CD34	Probably for cellular interaction and adhesion	Down-regulation (full-length form) during monocytic differentiation of M1 cells (Fackler *et al.*, 1995)	Decreased expression essential for terminal differentiation (Enforced gene expression) Enforced expression able to initiate monocytic differentiation up to intermediate stage (Enforced gene expression) Essential for progenitor formation in embryonic and adult hematopoiesis (Transgenic studies) (Fackler *et al.*, 1995; Cheng *et al.*, 1996)	Regulated by : c-*myb*. (reviewed in Hesketh, 1995)

1.3 Induced myeloid cell differentiation

1.3.1 Induced myeloid cell differentiation

Induced hematopoiesis has been observed in pathological processes, infections or treatment with cytotoxic drugs. This results in an increased production of cells largely restricted to a specific cell type in demand in response to an interplay of growth factors (CSFs and interleukins)

Induced myeloid cell differentiation can be mimicked *in vitro*, in primary culture initiated from normal bone marrow cells maintained by multiple growth factors. Alternatively, many myeloid leukemia cell lines, derived from hematopoietic cells at different stages of development and commitment, have been proved to be very useful tools for studying *in vitro* hematopoiesis. Very often, these cell lines are growth factor-dependent. They rely on the appropriate factor(s) for both survival and proliferation (factor-dependent cell lines, e.g. 32Dcl3). Some other leukemia cell lines, however, can produce or be engineered to produce the required growth factors themselves, acting in an autocrine manner. As autocrine growth factor production alone does not appear to play major roles in leukemogenesis / in the maintenance of the leukemic state, these leukemia cells often display response that reflect approximately the responses of normal cells from which the leukemia is derived. Like hematopoiesis *in vivo,* both primary bone marrow cell culture and leukemia cell line are able to be triggered to differentiate by certain growth factors (G-CSF), physiological inducers (retinoic acid, RA), pathogens or their derived products (lipopolysaccharide, LPS). Although leukemia cells cannot entirely represent the normal cells, they do retain some normal functions and responses of normal cells. Also, they are less vulnerable and easier to maintain, thus, providing us with very useful models for studying the properties of both normal and leukemia cells arrested at different stages.

Another flexibility of these cell lines is that they respond not only to different physiological growth factors but also to some non-physiological inducers (e.g. phorbol

12-myristate 13-acetate, PMA). It has been demonstrated that the same growth factor or non-physiological inducer can induce the differentiation of several cell lines to differentiate (e.g. G-CSF stimulates differentiation of WEHI-3B (D^+), M1 and HL-60 cells; PMA stimulates differentiation of U937 and WEHI-3B JCS cells). This suggests the cell lines are probably at similar stages of development, committed towards the same differentiation lineage, thus, receptive to the same inducing agent The same cell line can also be induced to differentiate by a repertoire of different physiological and non-physiological inducers but not the others (e.g. M1 cells can be induced to differentiate strongly by LIF but weakly by G-CSF while WEHI-3B(D^+) cells differentiate in response to G-CSF but not LIF). This probably reflects the characteristics of the cell line arrested at a certain degree of maturation and arisen under certain environments. Each of the cell lines retains certain inducible normal pathways for differentiation and is defective in some other pathways. In addition, the cells may respond differently to different inducers in switching on different pathways of differentiation towards either the same or different lineage (e.g. HL-60 cells can be triggered to undergo monocytic differentiation by PMA or Vit D_3 or granulocytic differentiation by GM-CSF, DMSO or RA) resembling the differentiation potential of normal cells. Therefore, by gathering the different responses of different leukemia cell lines developed under different environments and arrested at different stages, we may be able to assemble a scratch of all possible responses and differentiation pathways of the normal cells during hematopoiesis.

1.3.2 WEHI-3B JCS cells

WEHI-3B JCS is a recently isolated and characterized subclone of the WEHI-3B (D^-) line (Mak *et al.*, 1993). While WEHI-3B (D^+), WEHI-3B (D^-) and WEHI-3B JCS cells retained some common characteristics like producing IL-3 (Mak *et al.*, 1993), each of them has its unique characteristics and responses to different differentiation-inducing agent. For instance, WEHI-3B (D^+) but not WEHI-3B (D^-) nor WEHI-3B JCS cells was able to give granulocyte-macrophage colonies upon post-endotoxin serum stimulation (Mak *et al.*, 1993). Interestingly, although all of these lines express mRNA for G-CSF receptor (Mak *et al.*, 1993, authors' unpublished observation), only WEHI-3B (D^+) cells showed cell differentiation towards granulocyte-macrophage upon G-CSF induction. Both WEHI-3B (D^-) and WEHI-3B JCS cells were unresponsive to the factor (Mak *et al.*, 1993).

On the other hand, WEHI-3B JCS is distinct in its ability to differentiate towards the monocytic lineage when incubated with non-cytotoxic concentration of TNF-α (600-1200 U/ml) (Mak *et al.*, 1993). The cells also showed monocytic differentiation triggered by inducing agent like phorbol 12-myristate 13-acetate (PMA) (Mak *et al.*, 1993), lipopolysaccharide (LPS) (Chan *et al.*, 1995) or growth factors such as IL-1α or IL-1β (Chan *et al.*, 1995). Synergistic actions of growth factors to induce differentiation of JCS cells were also observed. Sub-optimal concentrations of TNF-α (50 U/ml) and IL-4 (100 U/ml) were able to induce monocytic differentiation of JCS cells (Leung *et al.*, 1994). Similar interaction was also observed when the cells were treated with TNF-α (20 U/ml) plus IFN-γ (300 U/ml) (Mak *et al.*, 1993).

Differentiation of JCS cells and the degree of maturation have been monitored by some or all of the following methods. In general, JCS cells had significant morphological changes after 3-4 day incubation with the above mentioned inducers. There was an increase in cytoplasm to nucleus ratio, cell size and vacuolation (Mak *et al.*, 1993, Leung *et al.*, 1994). Flow cytometry analysis indicated that the expression of differentiation antigens such as Mac I (Mak *et al.*, 1993; Leung *et al.*, 1994) , F4/80

(Leung *et al.,* 1994) and FcR (Mak and *et al.,* 1993) were increased while that of J11d was decreased (Mak *et al.,* 1993) as JCS cells were driven towards the monocytic lineage. Phagocytic activity demonstrated by the ability to engulf yeast particles was also increased as JCS cells differentiated (Mak *et al.,* 1993, Leung *et al.,* 1994). Functional assays investigating the NBT-reducing ability of the cells and non-specific esterase activity using fluorescein diacetate as substrate were also increased upon induction of JCS monocytic differentiation (Leung *et al.,* 1994). Increase in percentage of plastic adherent cells is an another indication of differentiation (Leung *et al.,* 1994). Triggering by appropriate differentiation-inducing agents as mentioned above, the clonogenicity (the ability to form colonies) (Mak *et al.,* 1993; Leung *et al.,* 1994) and tumorgenicity of the JCS cells (the ability to cause fatal leukemia in mice after intra-peritoneal injection of the treated cells) (Leung *et al.,* 1994, Mak *et al.,* 1994) were found to be decreased.

Pertussis toxins (PTx), which have been reported to interfere with the ADP-ribosylation of G proteins, were found to augment the effect of sub-optimal concentration of TNF-α (25 U/ml) (Mak *et al.,* 1994). Besides, preliminary findings suggested that prostaglandin E_2 (PGE_2) and cAMP, whose synthesis and accumulation respectively could be stimulated by PTx, were also capable of augmenting the TNF-α induced monocytic differentiation (personal communication with Dr. K.N. Leung). This enhanced effect of PTx was not limited to TNF-α–induced monocytic differentiation but could also apply to LPS or PGE_2-stimulated monocytic differentiation by JCS cells (Mak *et al.,* 1993, authors' unpublished data). Taken together, PTx-sensitive G proteins may be involved in the signaling pathway of JCS cell differentiation initiated by various agents (Mak *et al.,* 1994).

Interplay of cytokines may also be involved in the differentiation of JCS cells. It has been shown that TNF-α or IL-1β–mediated monocytic differentiation could only be blocked by TNF-α or IL-1β neutralizing antibodies respectively (Mak *et al.,* 1993; Chan *et al.,* 1995). Interestingly enough, the action of IL-1α on JCS cell differentiation was able to be blocked by both IL-1α and IL-1β neutralizing antibodies. In addition, during both LPS and TNF-α induced differentiation, there

was a significant up-regulation of IL-1α, IL-1β, TNF-α and IFN-γ gene expressions. Several conclusions can be drawn from these results. First, the differentiation-inducing action of IL-1α was mediated via the action of IL-1β during JCS cell differentiation. Second, the action of TNF-α on JCS cell differentiation may not be confined to direct signaling mechanism; TNF-α may drive the cells to differentiate via the production of other cytokines which can either work by themselves like IL-1α, IL-1β or work in synergy with TNF-α, like IFN-γ (Mak *et al.,* 1993) in an autocrine manner. Third, TNF-α may form a positive auto-regulation loop to enhance its action in stimulating differentiation of JCS cells (Chan *et al.,* 1995).

Besides cytokines or bacterial products, the effects of two distinct classes of molecules, flavonoids and benzodiazepines, on JCS cell differentiation are currently investigated. Plant flavonoids like nobiletin and tangeretin (methoxylated flavones) have been identified from the extracts of the pericarpium of *Citrus reticulata* (cv Jiao Gan) which are capable of inducing JCS cell differentiation towards macrophages and granuolcytes (Mak *et al.,* 1996). Biochanin A, belonging to the subclass isoflavones of flavonoids, drive JCS cells towards the monocytic lineage (personal communication with Dr. N.K. Mak). In contrast to biochanin A and other defined inducers of JCS cells reported so far, midazolam, a member of the benzodiazepines, led the cells towards the granulocytic pathway in addition to the monocytic pathway (personal communication with Dr. N.K. Mak).

1.3.3 Chemical Inducers -- Flavonoids and benzodiazepines

Flavonoids and biochanin A

Main classes of flavonoids

Flavonoids can be synthetic or natural-occurring. Over 4000 chemically distinct flavonoids have been identified in various plant sources (fruit, pollen, roots and heartwood). Flavonoids are also widely consumed in food of plant origin and the amount can be up to 1g daily. The compound is a benzo-γ-pyrone derivative taking the form of C_6-C_3-C_6 . They comprise a benzene ring A and six-member ring C carrying a substituent phenyl ring B either in the 2-position (flavonoid) or the 3-position (isoflavonoid). Flavonols and flavanones differ from each other by a hydroxyl group in the 3-position and a C_2-C_3 double bond. Ring C could either be a γ-pyrone or its dihydro-derivative in flavonols and flavonones (Figure 1.5). Flavonoids may occur as aglycons (the basic form), glycosides (with sugar residues) or methylated derivatives (Havsteen, 1983; Das, 1994).

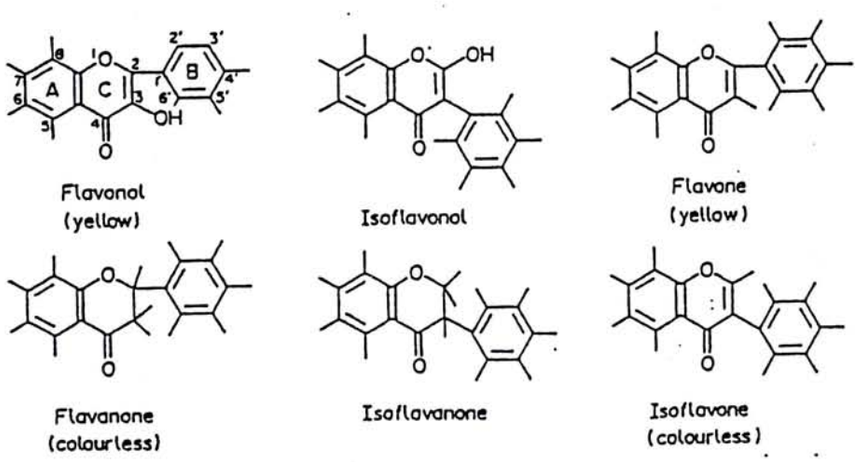


Figure 1.5. **The main classes of flavonoids.** The basic structure contains a benzene ring A and a six-member ring C carrying a ring B either in the 2-position (flavonoid) or the 3-position (isoflavonoid). Flavonols and flavanones differ from each other by a hydroxyl group in the 3-position and a C_2-C_3 double bond (Havsteen, 1983; Das, 1994).

Isoflavones and their distributions

The isoflavonoids are a large and distinct subclass of the flavonoids (Dewick, 1993). However, their distributions are almost limited to the subfamily Papilionoideae of the leguminosae. Only a few plants in the subfamilies Caesalpinioideae and Mimosoideae of the Leguminosae (beans) have been reported to contain isoflavonoids. A number of non-legume dicotyledon families are known to produce isoflavonoid derivatives; they are found in isolated plants or genera with more limited range of structures. Iridaceae is the major source of isoflavonoids among the monocotyledons while *Juniperus* and *Podocarpus* are known to produce isoflavonoids among the gymnosperms. A moss (*Bryum capillare*) has also been reported to produce several isoflavonoids and oligomeric derivatives. Marine coral (*Echinopora lamellosa*) is also a producer of isoflavonoids. Microorganisms and mammals fed with leguminous materials also produce isoflavonoids (reviewed in Dewick, 1993).

Isoflavones constitute the largest group of natural isoflavonoids. One of the members is biochanin A (5',7'-dihydroxy-4'methoxyisoflavone) (Figure 1.6). Biochanin A was found in a number of plant sources. These include cotyledon, seed and cell suspension culture of *Cicer arietinum* (chickpea plant), root of *Cicer mongoltavicum*, root, leaf and flower of *Dalbergia sissoides*, branch of *Dalbergia volubilis*, seed of *Glycine max,* stem of *Milletiia dielsiana*, *Ononis spinosa,* wood gall of *Wisteria brachybotrys* (reviewed in Dewick, 1993) and *Trifolium pratense* L. (red clover) (Cassady *et al,* 1988).

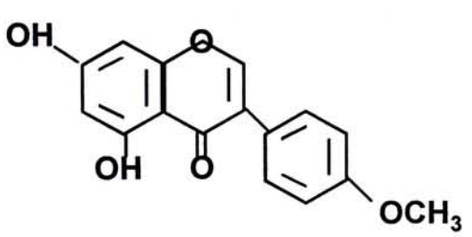


Figure 1.6. **Structure of biochanin A.**

Isoflavones and their biological activities

Flavonoids consist of a vast number of members and mediate a lot of biological and pharmacological actions. Many of them are potent antioxidants. They are also anti-inflammatory, anti-allergic, anti-hemorrhagic, anti-toxic and hepatoprotective. Their endocrine effects and effects on smooth muscles were also noted. They inhibit as well as induce several important enzymes (reviewed in Middleton and Kandaswami, 1993; Stavric and Matula, 1992). Isoflavones share some of these properties and one of the prominent member, genistein is well-known for its inhibition on protein tyrosine kinase (PTK) and topoisomerase. Many biological effects of genistein can be attributed to its activity on protein tyrosine kinase. Inhibition of T cell and mononuclear cell proliferation, production of IL-2 and various effects on the signal pathway are some of the examples (Middleton and Kandaswami, 1993). Besides, the compound mediates activities that are unrelated to its effect on PTK. Genistein or daidzein (another member of isoflavones) was able to reduce the platelet response while genistein or biochanin A could inhibit Epstein barr virus (EBV) activation (reviewed in Middleton and Kandaswami, 1993). Isoflavones also affected the endocrine systems. Genistein inhibited IL-1-stimulated PGE_2 synthesis and the induction of PG endoperoxides. They (e.g. formononetin) can also act as phytoestrogens causing infertility syndrome of sheep (reviewed in Middleton and Kandaswami, 1993).

Apart from these biological actions, we are interested in the cancer-related activities of isoflavones. The cytostatic effect of isoflavones (genistein) has been demonstrated in human T lymphocytic leukemia cell line MOLT-4 and HL-60 (Hirano *et al.*, 1994). These compounds also inhibited the mitogen-induced blastogenesis of lymphocytes (Hirano *et al.*, 1994). Moreover, genistein inhibited growth factor-induced mitogenesis of mouse 10T1/2 fibroblasts, rat liver T51B cells and the ras-transformed NIH 3T3 fibroblast cells (reviewed in Middleton and Kandaswami, 1993). Isoflavones have shown notable differentiating effect on different cell lines. For instance, genistein was able to induce the differentiation of TS12 and SJNKP neutroblastoma cell lines (Rocchi *et al.*, 1995), HL-60 (Constantinou *et al.*, 1990; Jing

and Waxman, 1995; Watanabe *et al.,* 1991) and K562 cells (Watanabe and *et al.* 1991). Another isoflavone, daidzein was also a growth inhibitor and potent differentiation inducer of erythroleukemia cells (Jing and Waxman, 1995).

Biochanin A and its biological activity

Biochanin A has been found in soy-based diet which is associated with lower incidence of tumor formation. It is also one of the active compounds in *Trifolium pratense* L. (red clover) described as the 'Top ten herbs in medical practice' in Medical Herbalism : A clinical newsletter for herbal practitioner Vol 6 Spring 1994. *Trifolium pratense* L. or the red clover was also believed to be a cure for various types of cancer fifty years ago (Hunt, 1994) and has been used in treatment of breast and ovary cancer (Mills, 1994) in addition to its alterative and expectorant effect. The extract of the plant was recently found to be anti-carcinogenic and the active component was biochanin A (Cassady *et al.,* 1988). The anti-carcinogenic effects of isolated biochanin A was also studied. The compound decreased the metabolism and DNA-binding ability of benzo[a]pyrene (a carcinogen) in V79 hamster embryo cell culture (Cassady *et al.,* 1988; Chae *et al.,* 1991, 1992). Anti-mutagenic activity of biochanin A in *Salmonella typhimurium* against two carcinogens, 2-aminoanthracene (Wall *et al.,* 1988) and aflatoxin B_1 (Francis *et al.,* 1989a) and one direct-acting carcinogen, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Francis *et al.,* 1989b) was also observed. Biochanin A is a growth inhibitor of MCF-7 and MCF-7-D-40 human breast cancer cells (Peterson and Barnes, 1991), HSC-45M2 and HSC-41E6 (Yanagihara *et al.,* 1993) gastrointestinal cancer cells and erythroleukemia MEL (Jing and Waxman, 1995). Besides, the compound is an inhibitor of tyrosine protein phosphorylation (Besterman and Schultz, 1990) which is frequently involved in cell proliferation and /or differentiation. In addition to anti-tumor effects, biochanin A can also act as phytoestrogen inhibiting male copulating behavior in Japanese quail (*Coturnix coturnix*) (Man and Peeke, 1982) and increasing progesterone synthesis in bovine granulosa cells (Kaplanski *et al.,* 1981). However, whether such estrogenic property is related to the involvement of estrogen receptor in inhibiting the growth of

human breast cancer cells is unknown (Peterson and Barnes, 1991). Finally, biochanin A also showed hypolipidemic activity in rats (Sharma, 1978, 1979).

Although growth inhibitory effect of biochanin A in different cell lines has been shown, the effect was cytostatic at low concentration and cytotoxic at high concentration (Yanagihara *et al.*, 1993). As far as we know, the differentiation-inducing effect of biochanin A (an isoflavone) was first demonstrated in JCS cells (personal communication with Dr. N.K. Mak). Hence, the gene expression it triggered is of much interest.

Benzodiazepines and midazolam

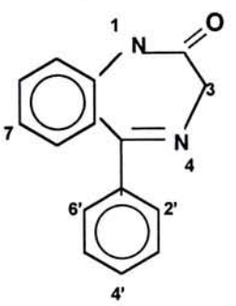
Major classes of benzodiazepines and midazolam

Benzodiazepines are widely prescribed drugs for their hypnotic, anxiolytic, anticonvulsant, muscular relaxant and amnesic actions. Their general structures are shown in Figure 1.7. The drugs can be classified according to their metabolic paths in the body into 'pro-nordiazepam' compounds, nitro-compounds and 'short-acting' compounds (Bellantuono *et al.*, 1980). 'Pro-nordiazepam' benzodiazepines such as diazepam (Figure 1.7) and medazepam yield nordiazepam or its halogenate homologues by hepatic N-dealkylation. The N-dealkyl derivatives are usually hydroxylated to oxazepam or its halogenated homologues. Finally, they are conjugated with glucuronic acid and excreted. Nitro-benzodiazepines like clonazepam (Figure 1.7) have no known active metabolites. Reduction of the nitroso group and acetylation are involved in the metabolic pathway. 'Short-acting' benzodiazepines like lorazepam (Figure 1.7) have no active metabolites and directly conjugated with glucuronic acid for excretion. Benzodiazepines can also be classified according to their binding activities to different receptors. Central benzodiazepine receptors (CBR) are located in the CNS and coupled with γ-aminobutyric acid (GABA) receptors and Cl^- channels. It has been suggested that the therapeutic effects mentioned above are mediated through these receptors. Peripheral benzodiazepine receptors (PBR) differ from CBR that they did not couple to GABA receptors and

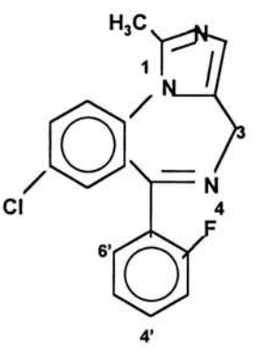
distributed in neuronal as well as non-neuronal tissues. As opposed to CBR, PBR do not have stereochemical preferences in binding (Parola *et al.,* 1993). CBR-specific ligand like clonazepam binds at high affinity with CBR but very low affinity with PBR. On the contrary, Ro5-4864 (4'-chorodiazepam) binds specifically to PBR. Lastly, there are some compounds like diazepam that bind both CBR and PBR with similar affinity.

Midazolam (8-Chloro-6-(2-flurorphenyl)-1-methyl-4H-imidazo[1,5-a] [1,4] benzodiazepine) is a short-acting derivative of diazepam. It is a water soluble benzodiazepine having a shorter duration action than diazepam. It is anxiolytic, sedative and currently used in anesthesia. It inhibits the rise of ACTH and corticosterone (Freire-Garabal *et al.,* 1992). It decreases arterial pressure without changing the heart rate. It is also a respiratory depressant.

Basic structure



Midazolam



Benzodiazepine	1	3	7	2'	4'	6'	Classification
R05-4864	CH_3	H	Cl	H	Cl	H	PBR-specific ligand
Diazepam	CH_3	H	Cl	H	H	H	'Pro-nordiazepam' compounds
Clonazepam	H	H	NO_2	Cl	H	H	Nitro-compounds
Oxazepam	H	OH	Cl	H	H	H	Short-acting compounds
Lorazepam	H	OH	Cl	Cl	H	H	Short-acting compounds

Figure 1.7. **Structures of the benzodiazepines and some selected compounds.** Benzodiazepines can be classified into 'pro-nordiazepam'compounds, nitro-compounds and short-acting compounds according to their metabolisms. There are also compounds that bind specifically to peripheral benzodiazepine receptors (Bellantuono *et al.,* 1980).

Biological action and therapeutic use

Benzodiazepines are employed in the treatment of insomnia (e.g. loprazolam), anxiety (diazepam), epilepsy (clonazepam), motor disorders, acute psychoses and used in anesthetic practice (midazolam). The drugs are able to suppress the noradrenergic and/or serotonergic pathways. They suppress activities in many limbic and other brain areas, decreasing the turnover of neurotransmittors in these areas. They also change the sleep pattern (Ashton, 1994), alter the neuroendocrine and autonomic nervous system (Schlumf *et al.,* 1992). Besides, the drugs have actions on other non-neural tissues (reviewed in Gavish *et al.,* 1992). Notably, diazepam stimulated human monocytic chemotaxis (Ruff *et al.,* 1985), broke down the cytokine network (TNF-α, IL-2 and IL-6) (Schlumpf *et al.,* 1992) and induced persisting depression of cellular immune responses by prenatal exposure (Schlumpf *et al.,* 1988). In addition, diazepam was found to inhibit synthesis of myosin heavy chain in cultured muscle cells and decreased cardiac muscle contractility (reviewed in Gavish *et al.,* 1992). The drugs like diazepam and Ro5-4864 can also modulate other cardiac functions. While diazepam can increase corticosterone and testosterone secretion, both diazepam and Ro5-4864 induce P_4 and E_2 secretion in placental explants (reviewed in Gavish *et al.,* 1992). Finally, induction of ornithine decarboxylase was also observed (Morgan *et al.,* 1985).

Benzodiazepines and tumor

Besides the biological actions and therapeutic effects mentioned above, benzodiazepines modulate proliferation and/or differentiation of some tumor cell lines. Diazepem (Valium), medazepam, lorazepam and to a lesser extent the CBR-specific ligands imposed a cytostatic effect in a dose dependent manner on Swiss 3T3 cells(Clarke and Ryan, 1980). Peripheral type benzodiazepines Ro5-4864 (4-chlorodiazepam) and diazepam inhibit growth of human lymphocytes and lymphoma cell line CCRF-CEM (Alexander *et al.,* 1992) as well as DNA synthesis of mouse spleen lymphocytes (Pawlikowski *et al.,* 1988). Anti-proliferative effect mediated by

various benzodiazepines was also found in thymoma cell AKR (cytostatic effect by Ro5-4864) (Wang *et al.,* 1984a), B103 and B104 neuroblastoma and Friend erythroleukemia cells (Wang *et al.,* 1984a, authors' unpublished data). Moreover, peripheral type benzodiazepines inhibit NGF-induced neurite outgrowth in PC12 cells in a dose-dependent manner (Curran and Morgan, 1985; Morgan *et al.,* 1985) and accelerate the onset of melanogenesis of melanoma B16/C3 (Matthew *et al.,* 1981). Cell differentiation of Friend erythroleukemia cells (DS19FEL) and HL-60 (Ishiguro *et al.,* 1987) induced by benzodiazepines were observed (Wang *et al.,* 1984b).

Having observed the biological actions of benzodiazepine on immune systems and its effects on tumor cell lines, we should not be surprised to see the differentiating effect of midazolam on the leukemia cell line JCS. However, reports on differentiating effects of benzodiazepines are very limited and there are none for midazolam. Also, midazolam drives JCS cells to a different lineage preference from the previously known inducers (personal communication with Dr. N.K. Mak); it is our interest to study the molecular mechanism of the differentiation process.

1.4 The aim of study

Differentiation commitment occurred at different points during hematopoiesis. Multi-potent stem cells reduce/abolish a pre-existing capacity for self-generation and produce committed progenitors restricted to a limited number of cell lineages (or more often to a single lineage). In bipotent granulocyte-macrophage progenitor cells, the commitment limits the cells to form only the granulocyte or macrophage progeny. Such commitment can be reproduced in leukemia cell model like WEHI-3B JCS which upon induction differentiate towards the granulocytic or monocytic pathway with observable phenotypes. Precise control of hematopoiesis is important for normal function of the blood system and preclusion of disorders (leukemia). With the help of a leukemia cell model, we are able to dissect the control mechanism of cell differentiation and/or proliferation. Such investigation will be rewarding in understanding both normal hematopoiesis and leukemic situation.

WEHI-3B JCS cell line was developed only recently and many 'proposed' inducers have been tested to accumulate more basic information about the cell line. Among these inducers, biochanin A and midazolam drove JCS cells into monocytic and monocytic/granulocytic differentiation respectively. Previous induction of WEHI-3B JCS cells by TNF-α resulted in endogenous production of various cytokine mRNA. These cytokines (IL-1α and IL-1β) not only expressed at high levels during the TNF-α stimulated differentiation but also directed cell differentiation of JCS cells themselves. In particular, the action of IL-1α was proved to be indirect and mediated through the activity of IL-1β (Chan *et al.*, 1995). Consistent with these findings, cytokines have also been shown to be important participants in myeloid cell differentiation. Hence, studying cytokine gene expression should give us insight into the mechanism of JCS cell differentiation. As biochanin A and midazolam represent two distinct types of molecules and are entirely different from TNF-α, it is anticipated that different differentiation pathways may be triggered by each of them. While both TNF-α and biochanin A lead JCS cells to monocytic lineage only, midazolam allows the production of granulocytic cells in addition to monocytic cells. These different

lineage commitment are likely to be controlled by different genes (including cytokines). On the other hand, biochanin A and TNF-α–induced JCS cells have the same lineage preference; thus, there may be some genes including cytokine genes that are commonly involved in both differentiation process. To examine and to compare the cytokine expression pattern triggered by biochanin A or midazolam in JCS cells, we employ a semi-quantitative protocol using reverse-transcription-polymerase chain reaction, cycle titration and specific hybridization. The protocol is simple, fast, flexible and more sensitive than conventional methods.

The role of cytokines in differentiation remain controversal; evidence has been accumulated to question the influence of cytokines on both differentiation commitment and hematopoiesis. With a view to delineate the molecular mechanism of myeloid cell differentiation, we are interested in studying the possible involvement of different types of genes in myeloid cell differentiation.

Midazolam is a very unique inducer of JCS cells; it triggers the cells to enter both the monocytic and granulocytic lineages. As no studies on granulocytic differentiation of JCS cells have been done, we started by searching genes that change in mRNA levels during the differentiation process stimulated by midazolam. Different approaches like differential screening and subtractive hybridization can be used for the purpose but it is the technique RNA fingerprinting by arbitrarily-primed PCR (RAP-PCR) that allows a time course study. Genes that are up-regulated or down-regulated during differentiation can also be identified at the same time. The protocol is fast, simple and requires a small amount of starting RNAs. The initially obtained short fragments representing differentially regulated genes, can be used as probes to obtain full cDNA sequences. Effective screening system is necessary to remove false-positives (genes later proved not to be regulated during the differentiation) so that at least a couple of differentially expressed genes during the differentiation can be revealed at last (proved by RT-PCR). These isolated genes may belong to characterized genes or novel genes. To extend our understanding of these isolated genes, we propose to study at the same time the expression profile of these isolated genes during biochanin A-induced JCS cell differentiation and during embryo

development. This allows us to examine the gene in normal tissue as well as to speculate the function of the novel genes in embryo development and differential effects of the isolated genes on different lineages.

The differentially regulated genes (in the form of short fragments) resulted from the current study need further characterization. One of our final goal is to find out the causative genes that direct the myeloid differentiation and/or bias the lineage-specific differentiation of JCS cells.

Chapter Two Cytokine Expression in Biochanin A and Midazolam-treated JCS cells

2.1 Introduction

2.1.1 Cytokine and myeloid differentiation

As discussed earlier, cytokines are the main regulators of hematopoiesis. In addition to the colony-stimulating factors GM-CSF, M-CSF, G-CSF and multi-CSF (IL-3) which are particularly important in myeloid cell differentiation, various interleukins, tumor necrosis factors and interferons have been reported to (1) modulate the growth and differentiation of the progenitor cells, (2) exert anti-proliferative activities on myeloid leukemia cells *in vitro* or (3) bias the lineage choice of the differentiating cells. Synergistic and negative effects of cytokines were also observed. In this section, we will concentrate on several cytokines that have previously been studied in JCS cells; their actions on bone marrow cells and other myeloid leukemia cells are summarized below.

Interleukin-1 (IL-1) has many biological activities in immune and inflammatory responses (reviewed in Dinarello, 1994). It is produced by a variety of cell types including hematopoietic progenitor cells (Watari *et al.,* 1996), stimulated monocytes/macrophage and induced myeloid cell lines such as U937 and HL-60 (Giavazzi *et al.,* 1995; Hass *et al.,* 1991; Mori *et al.,* 1992; Nishida *et al.,* 1988). It affects hematopoietic stem/progenitor cells directly or indirectly through potent factors produced by bone marrow stromal cells or other cell types (Ikebuchi *et al.,* 1988). It acts synergistically with M-CSF, GM-CSF, IL-3 and SCF (Heimfeld *et al.,*1991; Jacobsen *et al.,* 1994) to stimulate proliferation and development of stem cells and progenitors. *In vivo,* the molecule increases the circulating neutrophils, multipotent and committed granulocyte-macrophage precursors (reviewed in Whetton, 1990). The molecule also induced differentiation of myeloid leukemia cells indirectly

(Lotem and Sachs, 1989) or in co-operation with TNF-α *in vitro* (Weinberg *et al.,* 1992). Differentiation effect of the molecule (IL-1α) was also found *in vivo* (Lotem and Sachs, 1992). Lastly, mitogenic effect on early stage leukemia cell line and hematopoietic progenitors were also observed (Sakai *et al.,* 1987; Silvennoinen and Hurme, 1990).

Interleukin-4 (IL-4) has both stimulatory and inhibitory actions on myeloid cells (Kasukabe *et al.,* 1994). While it enhanced the granulocytic and monocytic colony formation induced by M-CSF and G-CSF respectively, it inhibited those triggered by IL-3 *in vitro* (Rennick *et al.,* 1987). Regarding the effect on myeloid leukemia cells, IL-4 influenced the balance between self-renewal and differentiation of leukemic stem cells (Miyauchi *et al.,* 1991). In addition, IL-4 alone either stimulated the growth of chronic myelomonocytic leukemia (CMMoL) cells after transformation (Yanagisawa *et al.,* 1995) or inhibited the growth of JCS, M1, FDCP-1 and 32Dcl-23 cells (Leung *et al.,* 1994, authors' unpublished data). It triggered monocytic differentiation of myeloid leukemia cell line (Imai *et al.,* 1991), co-operated with other cytokines like TNF-α to suppress the proliferation (Totpal and Aggarwal, 1991) and initiated the differentiation of myeloid leukemia cells (Leung *et al.,* 1994). Besides the proliferation-inhibitory and differentiation-inducing effects, IL-4 together with IFN-γ may also specify the cell lineages (Snoeck *et al.,* 1996).

Tumor necrosis factor-α (TNF-α), like IL-1, mediates a lot of actions in inflammation and immune responses. The two cytokines also share similar signal pathway (Verma *et al.,* 1995). TNF-α is produced in monocytes/macrophage, bone-marrow cell-derived colonies (Witsell and Schook, 1992) and myeloid cell lines (Hass *et al.,* 1991). It suppresses the growth of myeloid progenitors and stem cells in general (Selleri *et al.,* 1995; Witsell and Schook, 1992). The effect of this suppression is enhanced in the presence of IFN-γ (Selleri *et al.,* 1995). TNF-α enhanced IL-3- and GM-CSF- but inhibited G-CSF and SCF-dependent (Rusten *et al.,* 1994a; 1994b) proliferation of immature hematopoietic progenitor cells. It induced the monocytic differentiation of myeloid leukemia cells (Bloch *et al.,* 1995). The molecule also co-operated with IL-1 or IL-4 (Leung *et al.,* 1994; Tamatani *et al.,* 1987) to induce

myeloid leukemia cell differentiation. The molecule in combination with IFN-γ switched the eosinophilic differentiation of EoL-1 to monocytic differentiation suggesting an action on lineage commitment (Nakajima *et al.,* 1995).

The role of leukemia inhibitory factor (LIF) on leukemia cells is partially explained by its name. LIF is a potent inducer of M1 cell differentiation (Lord *et al.,* 1991). It also stimulated the differentiation of U937 in combination with ara-C or GM-CSF (Brach *et al.,* 1990, 1993). Besides, LIF prolonged the doubling time of some leukemia cell lines (Wang *et al.,* 1990). In the presence of GM-CSF or G-CSF it reduced the colony formation of HL-60 cells (Maekawa and Metcalf, 1989). Although LIF did not seem to affect the proliferation and/or differentiation of the most primitive stems cells (Leary *et al.,* 1990; Schaafsma *et al.,* 1992), it stimulated growth and proliferation of the multi-lineage, erythroid and eosinophil progenitor colonies (Verfaillie and McGlave, 1991). It also supported IL-3-dependent colony formation of blast colony-forming cells (Leary *et al.,* 1990).

All the cytokines mentioned above mediate their actions through specific receptors. These receptor genes may be developmentally regulated, induced upon stimulation (Dubois *et al.,* 1993) or restricted to myeloid cell lines (reviewed in Callard and Gearing, 1994). As a result, studying the cytokine and cytokine receptor gene expressions gives us insight into how these cytokines influence the JCS cells at different differentiation stages in an autocrine/paracrine manner.

2.1.2 Phenotypic studies biochanin A- and midazolam-treated JCS cells

Isoflavones and benzodiazepines are two distinctive groups of compounds. Each of them has distinguishing biological effects and therapeutic values as detailed in section 1.3.3. On the other hand, they both affect the growth and differentiation of tumor cells. Recently, the effect of two specific compounds, biochanin A and midazolam, members of isoflavones and benzodiazepines respectively were tested on the myeloid leukemia cell model WEHI-3B JCS (personal communication with Dr. N.K. Mak).

Both biochanin A (0-200 μM) and midazolam (0-40 μg/ml) suppressed the growth of JCS cells in a dose-dependent manner. Close to 100 % growth inhibition of JCS cells were achieved using either 200 μM biochanin A or 40 μg/ml midazolam as examined by ^{3}H-methylthymidine incorporation assay.

The two compounds also induced differentiation of JCS cells. Biochanin A at 50 μM or midazolam at 10 μg/ml increased the percentage of phagocytic cells from a few % to almost 40%. On close examinations, the differentiation conducted by the two compounds were different. While biochanin A drove the blast cells entirely to the monocytic lineage, midazolam led the cells to produce both granulocytes (neutrophils) and monocytes.

JCS cells started to show their differentiation characteristics after they have been incubated with 50 μM of biochanin A for 2 days. The percentage of blast cells decreased from 99.7 $\pm$1.2 % to 94.5 $\pm$ 3.0 %. In contrast, the percentage of cells at intermediate stage rose from 0.3 $\pm$1.2 % to 4.5 $\pm$ 3.0 % and that of the macrophage was also slightly increased from 0 to 0.8 $\pm$ 0.6 %. On day 3 after biochanin A incubation, the differentiating effect of biochanin A became obvious. The blast cell percentage dropped markedly from 96.2 $\pm$ 6.4 % to 79.2 $\pm$ 3.8 %. The proportion of cells at intermediate stage also increased significantly from 3.7 $\pm$ 5.8 % to 13.3 $\pm$ 2.1 % and that of macrophage increased from 0.2 $\pm$ 0.6 % to 7.3 $\pm$ 2.1%.

The effect of midazolam on JCS cells was different. After midazolam at 10 μg/ml was added to the cells for two days, the percentage of the blast cells dropped dramatically from 98.5 ± 0.5 % to 0.8 ± 0.8 %. The proportion of cells at intermediate stage, however, increased significantly after midazolam treatment from 1.5 ± 0.5 % to 56.7 ± 6.5 % and that of the macrophage increased from 0 % to 35.7 ± 5.3 %. Notably, 6.5 ± 1.5 % of metamyelocytes and 0.3 ± 0.6 % PMN (neutrophils) were found. On day 3 of midazolam incubation, the proportion of cells are predominantly macrophage (91.2 ± 4.9 %). No blast cells were found and a small percentage of cells at intermediate stage (8.0 ± 4.5 %) and metamyelocytes (0.8 ± 1.0 %) were observed. No more PMN could be detected in the population on day 3.

The different differentiation-inducing effect of biochanin A and midazolam were also shown in the expression of antigenic markers. The expression level of monocytic specific markers Mac I and F4/80 were up-regulated after JCS cells have been incubated with biochanin A for 3 days. Conversely, the expression level of a granulocytic marker Gr-1 was slightly down-regulated after the treatment of biochanin A. For JCS cells treated with midazolam for 3 days, the expression of Gr-1 (specific for granulocytic pathway) was enhanced in addition to that of Mac I and F4/80.

Taken together, biochanin A-induced cells were driven to an entire monocytic pathway while midazolam-induced cells were led to produce both granulocytes and macrophage.

Briefly, both biochanin A and midazolam were able to inhibit the growth and to induce differentiation of JCS cells as evidenced by morphological examination, differentiation marker expression and functional assays. The next important step is to find out the signaling pathway and/or mechanism that makes up the changes. Besides, JCS cells respond quite differently to biochanin A and midazolam in terms of lineage commitment. Thus, it will be very crucial to know what different genes did the two inducers switch on or switch off and how these differences in gene expression can be related to the differentiation and/or lineage commitment. We started by studying the mRNA levels of some chosen cytokines, those that have been found to be involved in JCS cell differentiation (Chan *et al.*, 1995). We aim to correlate the change of mRNA

levels of these cytokine genes with the differentiation program of JCS cells as stimulated by biochanin A or midazolam.

2.1.3 Cytokine regulation at transcriptional level

With the exception of some cell lines like WEHI-3B that constitutively produce IL-3, most of the cytokine mRNAs are undetectable in cells unless they are stimulated by proper inducers like infectious agent, mechanical injury or toxic stimulus. Thus, such genes are likely to be controlled at the transcriptional level. Most of the transcriptional machinery of cytokine genes are uncharacterized but it is anticipated that both positive and negative regulators, constitutive and inducible factors are required. For instance, constitutive factors like NF-κB as well as inducible factor AP-1 are involved in cytokine transcription while glucocorticoid hormone is a classical inhibitor of cytokine gene expression (for reviews, see Callard and Gearing, 1994; Taniguchi, 1988; Turner and Feldmann, 1990). Also, most of these cytokine genes (IL-1α, IL-1β, IL-2, IL-5, TNF, IL-6, IL-8 and TGFβ) represent early response genes peaked at 1 to 6 hours at least in activated blood mononuclear cells and T cells. These genes have half-lives ranging from 15 minutes to over 5 hours (reviewed in Turner and Feldmann, 1990) although exceptions are found in other cytokines like LIF which has a long half-life in activated monocytes (Anegon *et al.,* 1990). These cytokine genes can be induced to transcribe with or without *de novo* protein synthesis depending on different situations and different cell systems (reviewed in Turner and Feldmann, 1990).

Similar to previous investigation on cytokine gene expression, JCS cells treated with either biochanin A or midazolam for 18 hours (one cell cycle of JCS cells) and 46-48 hours (when differentiation phenotypes exhibit) were chosen for study in this investigation.

2.1.4 Cytokine mRNA phenotyping by a semi-quantitative approach

Semi-quantitative PCR

There are numerous methods for studying the level of mRNA expression, for example, Northern blots, RNA dot/slot blots, nuclease protection assays, nuclear run-on assays and *in situ* hybridization. In this report, we use a more recent method, reverse-transcription polymerase chain reaction (RT-PCR) to study the mRNA level of cytokines. Generally, three problems are encountered in the study of cytokine mRNA. As mentioned before, cytokine genes are not constituitvely expressed and the mRNAs are degraded rapidly after the induction is removed. Thus, expression at different intervals must be examined. Moreover, cytokine is usually expressed at low levels. Hence, a sensitive detection system is required. Conventional techniques like Northern blot represents a simple approach but lacks sensitivity in detecting cytokine mRNA as commented in Dallman *et al.* (1991). RNase protection assay is a more sensitive technique but large amount of mRNA is often required. Nuclear run-on assay gives a reasonably accurate measurement of mRNA transcription level at specific time point but again relatively large amounts of materials are required ($5X10^7$ cells per assay). *In situ* hybridization needs no RNA or DNA preparation from the cells and the spatial distribution of the mRNA can be determined. It is sensitive and the number of mRNA copies per cell can be estimated. In addition, it is readily combined with immunohistochemistry to detect the gene products simultaneously. Nevertheless, it is technically demanding.

The starting materials for reverse-transcription polymerase chain reaction are total RNAs (or cytoplasmic RNAs or mRNAs) which are converted into cDNAs. The cDNAs in turn are used as templates for PCR using gene-specific primers. This is a very sensitive method detecting genes of less than one part per 10^8 copies (reviewed in Mason *et al.,* 1993) or 0.003-0.1 copies per cell (reviewed in Kohler, 1995). Besides, the amount of samples needed for detection is much smaller. However, the method is not readily quantitative. Various techniques have been devised to correlate the initial amount of the gene under study with the amount of the final products or with the

number of amplification cycles. Either an internal (in the same tube as the target cDNA) or an external standard (in different tubes separate from the target cDNA) serves the purpose. Both internal and external standards can be exogenous or endogenous. Endogenous standards are usually house-keeping genes which do not change in the samples being compared. Exogenous standards are sequences that are artificially introduced and the amount added is precisely known. For external standards, tube-to-tube variations must be kept to a minimum and comparison must be made within the exponential phase of the PCR. Internal endogenous gene has different primer requirements and hence no competition with the target gene. On the other hand, the priming efficiency of the two genes may be different and the two genes may reach the plateau phase at different times. To solve the problem, exogenous gene having same primer requirement but differs in size or restriction site can be used. Competitive PCR is also an alternative in which increasing known amount of the exogenous competitive standard is added to replicate reactions containing the target cDNA. The concentration of the target cDNA is equal to the standard when the amounts of both amplification products are equivalent.

Cycle titration

In many cases, it may be sufficient to compare the relative amount of a particular gene in the samples. A simpler method was described in Dallman *et al.* (1991) and Dallman and Porter (1992) for this purpose. Aliquots of PCR products amplified from different cDNA samples were taken out at multiple PCR cycle numbers so that the plateau phase of the reactions could be monitored. The PCR products of different cycle numbers from different samples could then be analyzed by agarose gel electrophoresis followed by Southern blotting or dot-blotting. The PCR products blotted on the membrane were hybridized to an internal oligomers that lies within the target sequence between the two original primers. Using this method, 10-fold difference of the starting mRNA levels between samples could be detected at five PCR cycle intervals. Numerous reports have been successful in comparing the expression of cytokine genes using this method/principle (Dallman *et al.*, 1991; Murphy *et al.*, 1993)

Filter hybridization

To verify the PCR products obtained and to compare amount of PCR products from different samples at different PCR cycle numbers, Southern/dot blotting followed by filter hybridization is most suitable. To avoid the inconvenience and hazards posed by radioisotopes and to allow immediate detection and multiple exposures, non-radioactive labeling together with chemiluminescent detection is preferred. Among the non-radioactive labels, digoxigenin (DIG) occur specifically in the *Digitalis* plant and avoids non-specific binding caused by the use of ubiquitous vitamin like biotin. DIG system employs anti-DIG alkaline phosphatases which couple the DIG label with the chemiluminescent substrates. The system is also highly sensitive; it allows the detection of 0.1 pg of homologous DNA after Southern blotting (reviewed in Hughes *et al.,* 1995).

Hence, cycle titration and Southern/dot blotting followed by filter hybridization conform a convenient and sensitive method for studying cytokine gene expression in biochanin A- and midazolam-induced JCS cells.

2.2 Materials

2.2.1 Cell line

The murine myeloid leukemia cell line WEHI 3B (JCS) (Mak *et al.*, 1993) was cultured in RPMI 1640 supplemented with 10 % fetal calf serum (FCS, Gibco), 2 mM glutamine, and 50 U/ml penicillin, 50 μg/ml streptomycin, and 10 μg/ml neomycin. The cultures were maintained under a humidified atmosphere of 95 % air / 5 % CO_2 at 37°C.

2.2.2 Chemicals and buffers

1. Agarose type I : Low EEO	Sigma A6013
2. Biochanin A (5, 7-dihydroxy-4'-methoxyisoflavone)	Sigma D2016
3. Bromophenol blue λ_{max} 595 nm	Bio-Rad 161-0404
4. Cesium chloride	Sigma C4036
5. Diethyl pyrocarbonate, DEPC	Sigma D5758
6. Ethidium bromide	Sigma E8751
7. Ethanol, absolute	Ajax 214
8. Hydrochloric acid, 36%	Ajax 1367
9. Lauryl sulfate/ Sodium dodecyl sulfate, SDS	Sigma L5750
10. N-Lauroyl sarcosine	Sigma L5125
11. Lithium chloride	Sigma L8895
12. Magnesium chloride	Sigma M9272
13. Maleic acid	Fluka Chemika 63180
14. β-mercaptoethanol	Sigma M7154
15. Midazolam	Roche
16. Mineral oil	Sigma M5904
17. RPMI 1640	Sigma
18. Sodium chloride	Sigma S9625
19. Sodium citrate, trisodium salt	Sigma S4641

20. Sodium hydroxide	Sigma S5881
21. Synergel	Diversified Biotech Syn-100
22. Tris-acetate-EDTA (TAE), 25X liquid concentrate	Amresco 0796-1.6L
23. Tris-base	Boehringer Mannheim 604205
24. Tween 20	Bio-Rad EIA
25. Xylene cyanole FF λ_{max} 615 nm	Bio-Rad 161-0423

2.2.3 DIG system (Appendix A3)

1. DIG oligonucleotide 3'end labeling kit	Boehringer Mannheim 1362372
2. DIG luminescent detection kit	Boehringer Mannheim 1363514

2.2.4 Enzymes and nucleic acids

1. 1kb DNA ladder	GIBCOBRL 15615-024
2. M-MLV reverse transcriptase	GIBCOBRL 28025-013

- 5X reverse transcriptase buffer : 250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$,
- Dithiothreitol (DTT)

3. Oligo-dT_{12-18}	Pharmacia 7858-5A-260
4. Primer oligo (see also Table 2.1)	Integrated DNA technologies
5. rRNAsin	Promega N2512
6. Thermal prime plus polymerase	Advance Biotechnology AB301

- 10X reaction buffer : 200 mM $(NH_4)SO_4$,750 mM Tris-HCl pH 9.0, 0.1% Tween
- 25 mM $MgCl_2$

7. Ultrapure dNTP set 2'-deoxynucleoside 5'triphosphate minimal diphosphate	Pharmacia 27-2035-01

2.2.5 Solutions

1. Agarose gel, 2 %	2 % (w/v) agarose in 1X TAE buffer
2. Alkaline transfer buffer	1 M NaCl, 0.4 M NaOH
3. Blocking solution	1 X blocking reagent diluted by maleic acid buffer
4. Blocking stock solution	10 % (w/v) blocking reagent in maleic acid buffer
5. Detection buffer	0.1 M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5 (20 °C)
6. GT solution	4 M guanidinium thiocyanate, 0.1 M β-mercaptoethanol
7. Maleic acid buffer	0.1 M maleic acid, 0.15 M NaCl, adjusted to pH 7.5 (20 °C) with solid NaOH
8. Hybridization buffer	5X SSC, 1% (w/v) blocking solution, 0.1% N-lauroyl-sarosine, 0.02% sodium dodecyl sulfate
9. Standard saline citrate (SSC), 20 X	3 M NaCl, 0.3 M sodium citrate; pH 7.0
10. Washing buffer	maleic acid buffer, 0.3 % (v/v) Tween 20
11. Synergel, 1.5 %	1.5 % (w/v) Synergel, 0.7 % (w/v) agarose in 1X TAE buffer

2.3 Methods

2.3.1 Isolation of total RNA by guanidinium thiocyanate/cesium chloride isopycnic gradient

All the cell cultures used in this study were kindly maintained by Mr. Alex, W.H. Cheung and Mr. Bats, S.W. Wong in the Department of Biology, the Hong Kong Baptist University.

Cellular total RNA was obtained by guanidinium thiocyanate and cesium chloride gradient method (Chirgwin *et al.,* 1979).

Untreated JCS cells, cells treated with 10 μg/ml midazolam for 18 and 48 hours, and cells treated with 50 μM biochanin A or its solvent (0.25 % ethanol) for 18 and 46 hours were harvested and collected by centrifugation at 200 g for 5 minutes at room temperature. The supernatant was decanted and the cells were washed once in RPMI 1640. The cells were resusupended in 200 μl of RPMI 1640 and the suspension was added drop by drop to 4 ml (per 10^7 cells) GT solution under vortex. The resulting cell lysates of different samples were stored at -70 °C until use. Freezed cell lysates were recovered in a 65 °C water bath with constant vortexing until it is completely thawed. The thawed lysate was then squeezed through a 15 gauge needle 5 times by a sterilized syringe.

1 ml cushion of 5.7 M cesium chloride (CsCl) was pipetted into a 4.4 ml polyallomer ultracentrifuge tube (Bechman). The treated cell lysates were laid carefully on top of the CsCl cushion until the tube was completely filled. The preparations were centrifuged in a Bechman rotor SW60Ti at 32,000 rpm for 18 hours at 18 °C. At the end of the run, all the supernatant at the top (~1 cm from the pellets) was removed by aspiration. The tube was then quickly inverted and remained in its inverted position to drain out the remaining liquid. The most recalcitrant droplets were aspirated without disturbing the pellets at the bottom of the tube. The inverted tube was left air-dried for 5 minutes. The top portion of the tube was then cut off, at

about 0.7 cm from the bottom, with a sterile scalpel. Translucent RNA pellets were then dissolved in 40 µl at a time (in a total of 400 µl) of DEPC-treated water. The solution was transferred to a sterile 1.7 ml eppendorf tube each time and vortexed to ensure complete dissolution. The RNA solution was separated from insoluble contaminants, if any, by centrifugation at 14 000 rpm on a bench-top microcentrifuge for 1 minute at room temperature (Eppendorf). The RNA was then precipitated by 45 µl of 3 M sodium acetate and 1 ml of absolute ethanol. The mixture was centrifuged using a bench-top microcentrifuge at 14 000 rpm for 30 minutes at room temperature (Eppendorf). The precipitate was washed once by 70 % and once by 100 % ethanol. After being dried in a speed vacuum concentrator (Savant) for 10 minutes, the precipitate was resuspended in DEPC-treated water. The amount of RNA was estimated by spectrophotometry at 260 nm UV light and 1 absorbance unit was taken as an equivalent of 40 µg RNA.

2.3.2 Reverse-transcription polymerase chain reaction (RT-PCR)

The cytokine expression of uninduced and induced cells at different time points was assessed by reverse-transcription polymerase chain reaction (RT-PCR).

1 µg of total RNA isolated from each treatment of cells was heated to 65 °C for 10 minutes and reverse-transcribed at 37 °C for 1 hour by adding 10 µl master mix having 40 U RNase inhibitor, 0.1 µg oligo dT_{12-18}, 0.5 mM dNTP mixture, 1 X reverse transcriptase buffer, 10 mM DTT and 200 U Moloney murine leukemia virus (M-MLV) reverse transcriptase. This reaction could be scaled up for 5 µg total RNA per reaction by increasing the amount of each components in proportion.

After being heated at 94 °C for 10 minutes and chilled on ice for 3 minutes, 2 µl of the reverse transcription solution of each sample was amplified in a 50 µl PCR reaction containing 1.5 mM $MgCl_2$, 1X reaction buffer, 0.2 mM of each dNTP, 1 pmol/L of each primer and 0.25 U Thermoprime$^+$ DNA polymerase. All the components except the templates were pipetted from the same master mix for all

samples to be compared. The thermal cycle profile was : denaturation at 94 °C for 1 minute, annealing for 1 minute at temperature optimized for each pair of primers (Tm-10 °C, see Table 2.1) and extension at 72 °C for 1 minute.

The sequence of primer pairs, melting temperatures and the predicted sizes of the amplified PCR fragments are shown in Table 2.1.

The efficiency of RNA isolation and RT-PCR was confirmed by amplifying 2 μl of the same reverse transcription solution with GAPDH (glyceraldehyde-phosphate dehydrogenase)-specific primers for 20 cycles. GAPDH was also used as an external control for normalization among different samples. The PCR products, together with a molecular marker (1 Kb ladder), were loaded into a 1.5 % TAE Synergel and visualized under ethidium bromide staining (0.5 μg/ml).

Table 2.1 : **List of specific primer for various genes.**

Gene		Sequence (5' to 3')	Tm* (°C)	Product size (bp)
GAPDH	upper primer	ACC ACA GTC CAT GCC ATC AC	62	452
	lower primer	TCC ACC ACC CTG TTG CTG TA	62	
IL-1α	upper primer	ACA GTA TCA GCA ACG TCA AGC AA	66	546
	lower primer	CCG ACT TTG TTC TTT GGT GGC A	66	
	internal probe	GGC AAC TCC TTC AGC AAC ACG	66	
IL-1β	upper primer	GAG CTT CAG GCA GGC AGT ATC	66	382
	lower primer	GTA TAG ATT CTT TCC TTT GAG GC	64	
	internal probe	CAC TTG TTG GTT GAT ATT CTG TC	64	
IL-3	upper primer	AAG CTC CCA GAA CCT GAA CTC	64	205
	lower primer	TGA AGA CCC CTG GCA GCG CA	66	
	internal probe	ATT CGC AGA TGT AGG CAG GCA	64	
IL-4	upper primer	TGA CGC ACA GAG CTA TTG ATG G	66	422
	lower primer	ATG ATG CTC TTT AGG CTT TCC AG	66	
	internal probe	AGC TGG GGG TTG AGA CC	56	
TNF-α	upper primer	TCC CCA AAG GGA TGA GAA GTT C	66	411
	lower primer	TCA TAC CAG GGT TTG AGC TCA G	66	
	internal probe	CAC ACT CAG ATC ATC TTC TC	58	
LIF	upper primer	TCT CTT CAT TTC CTA TTA CAC AGC	66	415
	lower primer	GAC CAC CAC ACT TAT GAC TTG C	66	
	internal probe	CAC GGT ACT TGT TGC ACA GAC	64	
J11d	upper primer	GAC ATG GGC AGA GCG ATG GTG GCC AG	86	328
	lower primer	GGA CCT GTT TCT TCC TGA TCA CAT TGG AC	86	
	internal probe	AGA GAG AGC CAG GAG ACC AG	64	
IL-1RtI	upper primer	ATG GAA GGG ATG ACT ATG TTG GA	66	506
	lower primer	GCT GCA GCC TCT TAT GAT GGG	66	
	internal probe	TGA TCT CCG TTG GGC TGG CA	64	
IL1-RtII	upper primer	AAG GAA CAA CCA CGG AAC CCA T	66	446
	lower primer	AGC CCT GCG TTT ACA CCG TCT	66	
	internal probe	CGC AAT GCT CCA GGA GAA CGT	66	
IL-3R (AIC2A)	upper primer	TAC ACA CGA TTT TCT AAT GGA GAT A	66	326
	lower primer	TAG ATG CTG TTG GGT AGG AAT AG	66	
	internal probe	GAG CTG AAG GAG GAA CCT GAC	66	
IL-3R (AIC2B)	upper primer	ATA CAC GAT TTT CCA TCA CAA ACG	66	325
	lower primer	TAG ATG CTG TTG GGT AGG AAT AG	66	
	internal probe	GAG CTG AAG GAG GAA CCT GAC	66	
IL-3Rα	upper primer	TGC TCA TTC CAG TCG CTC TCC	66	560
	lower primer	AGG AGG CCA TGG TGG AAT CTG	66	
	internal probe	AGC CGT TGC ACT TCC ACT TGT CA	66	
LIF-R(S)#	upper primer	TCA TCA CCA CCT TCG AAA ATA GC	66	340
	lower primer	GCG CAA CAA TGG GAG CCA GT	64	
	internal probe	TGG TGC AAC TCA TCT CGG TCT	64	
LIF-R(L)##	upper primer	TCA TCA CCA CCT TCG AAA ATA GC	66	415
	lower primer	CCG CAT ATT TTA AGT GAC CGC T	64	
	internal probe	TGG TGC AAC TCA TCT CGG TCT	64	

*Tm : calculated from 2°C (A+T) + 4°C (G+C)
transmembrane form
soluble form

2.3.3 Southern blotting

PCR products were electrophoresed and stained with 0.5 μg/ml ethidium bromide in a 2 % TAE agarose gel. A photo of the gel (Polaroid 667) was taken under UV excitation with a fluorescent scale laid alongside the gel. The gel with the unused parts removed was depurinated in 0.25 M HCl for 10 minutes and briefly rinsed with distilled water. The DNA on the gel was transferred onto a positively-charged nylon membrane (Boehringer Mannheim 1209299) by upward capillary action (Figure 2.1) in 0.4 M NaOH for 4 hours. The gel was then stained in 1 μg/ml ethidium bromide for 30 minutes and examined under UV light to confirm a complete transfer of DNA. The nylon membrane was neutralized in 2 X SSC for 10 minutes, air-dried and baked for 30 minutes at 120 °C. The membrane was stored at room temperature until hybridization.

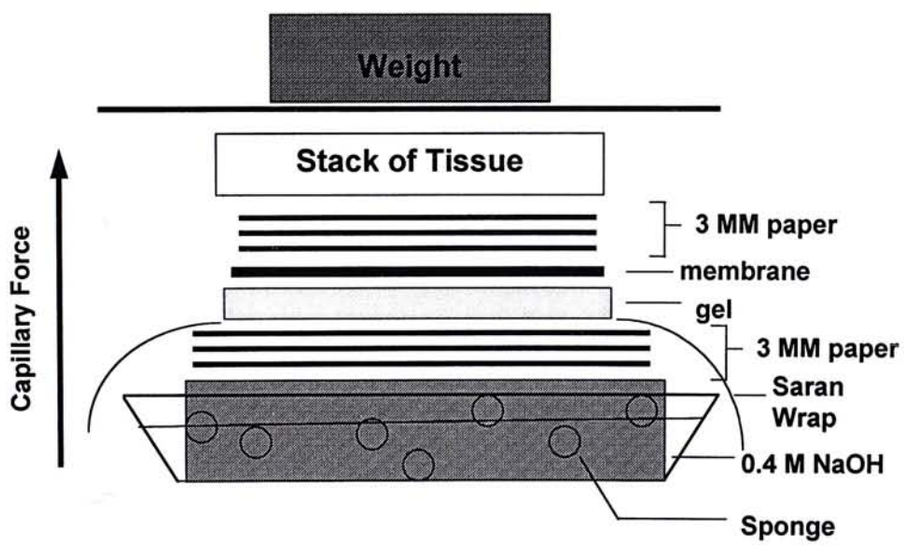


Figure 2.1. **Setup for Southern blotting.**

2.3.4 Cycle titration and dot blotting

Cycle titration / dot blotting is a semi-quantitative approach for studying the level of gene expression (Dallman *et al.,* 1991; Dallman and Porter, 1992). 6 identical

tubes of 50 µl PCR reaction of each sample under comparison were set up as described in section 2.3.2. One tube of each sample was removed from the PCR machine every five cycles from 10-35 cycles (or otherwise specified). The PCR products were then analyzed by dot blotting. A positively-charged nylon membrane (Boehringer Mannheim 1209299) cut to fit (10 X 13 cm) the BioDot microfiltration apparatus (Bio-Rad) was laid on the gasket of the apparatus with all holes covered. The apparatus was assembled as described by the manufacturer. Briefly, the sample template was put on top of the membrane following the glide pins. The sealing screws were tightened in a diagonal crossing manner (Figure 2.2). 200 µl of distilled water was applied to all sample wells to pre-wet the membrane while the vacuum was on. 10 µl of PCR products from each sample and cycle number was denatured in 0.2 M NaOH in a total volume of 200 µl solution for 15 minutes. The denatured products were applied to the wells and drained. Then, 400 µl of 20 X SSC was applied to each well and drained. The micro-filtration apparatus was dismantled in the presence of vacuum. The membrane was air-dried on a piece of blotting paper before baking for 30 minutes at 120 °C.

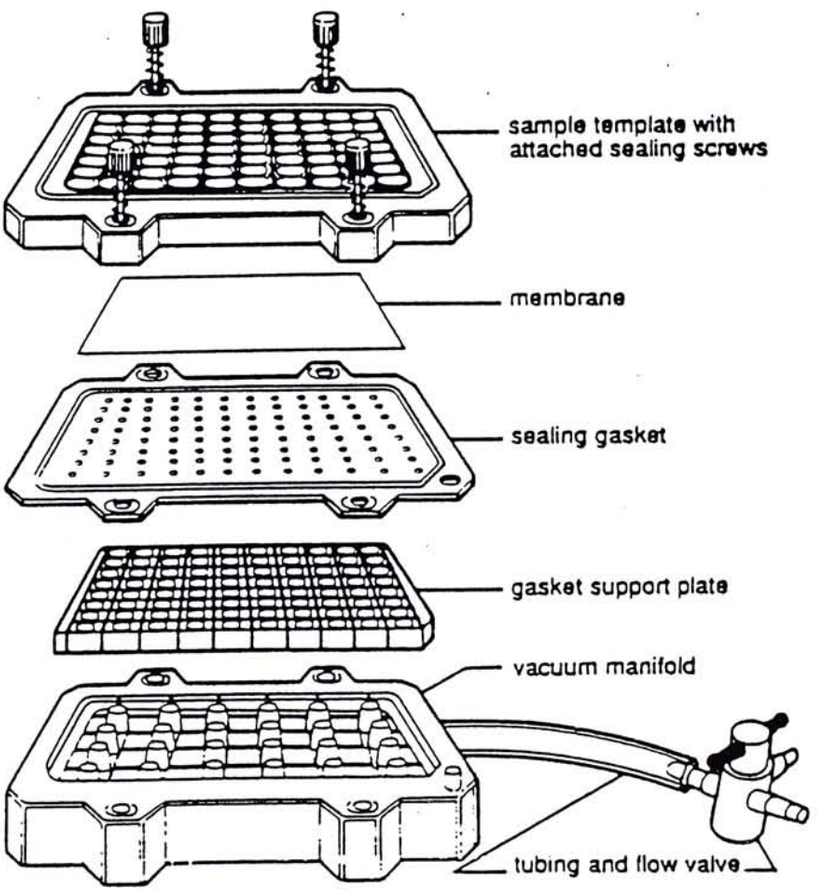


Figure 2.2. **Setup for dot-blotting.** (Reproduced from manufacturer's manual, BioDot Apparatus, Bio-Rad SF)

2.3.5 DIG 3' end labeling of probes

Oligonucleotides ranging from 16-22 nucleotides in length were labeled with digoxigenin-ddUTP (DIG-ddUTP) using the DIG oligonucleotide 3' end labeling kit (Appendix A3) and designated as internal probes (Table 2.1). For each probe, 100 pmol of oligonucleotides in 8 µl of DEPC-treated water was mixed with 12 µl of a master mix containing 1X tailing buffer, 5 mM cobalt chloride solution, 0.05 mM DIG-ddUTP and 50 U terminal transferase. The labeling reaction was done for 15 minutes at 37 °C and placed on ice immediately. 2 µl of 1 mg/ml glycogen in 0.2 M EDTA solution pH 8.0 was added to stop the reaction. The labeled oligonucleotides were precipitated by 2.5 µl of 4 M lithium chloride solution (LiCl) and 75 µl of pre-chilled (-20 °C) absolute ethanol, left for 2 hours at -20 °C and centrifuged for 30 minutes at room temperature. The pellets were carefully washed with 70 µl of cold 70 % ethanol, air-dried and dissolved in 50 µl of DEPC-treated water to a final concentration of 2 pmol/µl. The labeled probes were stored at -20 °C until use.

2.3.6 Hybridization and stringency wash

Nylon membranes having immobilized PCR products were put in 50 ml plastic centrifuge tubes (Falcon) and pre-hybridized with 20-25 ml of hybridization solution. The tubes were rotated constantly for 2 hours at hybridization temperature in a hybridization chamber. After hybridization, the hybridization solution was replaced with 5 ml of fresh hybridization solution added with 4 pmol/ml labeled probe. The hybridization was done for 12 hours at 10 °C below the melting temperature of the probes (Table 2.1). Membranes were then washed twice with 20-25 ml of 2 X SSC, 0.1 % SDS (w/v) followed by 0.5 X SSC, 0.1 % SDS (w/v) for 15 minutes at hybridization temperature. After that, the membranes were ready for signal detection.

2.3.7 Chemiluminescent detection

Chemiluminescent detection was done as described by the manufacturer using the luminescent detection kit (Appendix A3). The nylon membrane was first removed from the falcon tubes and rinsed for 5 minutes in a plastic box containing washing buffer. The membrane was then incubated in 100 ml of blocking solution for 30 minutes followed by 40 ml of diluted anti-DIG-AP conjugate (75 mU/ml) for another 30 minutes with constant mixing at room temperature. The membrane was subsequently washed twice in 100 ml of washing buffer for 15 minutes, equilibrated in 100 ml of detection buffer for 5 minutes and incubated in 40 ml of CSPD solution (120 X dilution) for 5 minutes at room temperature. After excess solution was dripped and blotted off, the damp membrane was sealed in 2 pieces of Saran wrap followed by incubation for 15 minutes at 37 °C. The sealed membrane was exposed to an X-ray film (Kodak X-OMAT AR) for 5-10 minutes. The membrane after detection was kept at 4 °C in sealed plastic bag.

2.3.8 Quantitation by densitometry

The image of the blots developed on the X-ray film was scanned using a GS-670 imaging densitometer (Bio-Rad) in transmittance mode. Each dot on the scanned image was individually selected and analyzed using the volume integration function in the software Molecular Analyst™ (Bio-Rad). The signal of each dot was presented as the integrated volume = [OD X area (mm^2)]. After a background region was identified, the value of the integrated volume was adjusted for global background removal and output as adjusted volume. To minimize the effect of non-uniform background, the command 'Local Background Subtraction' was performed so that the mean background value around the perimeter of each dot was subtracted. The quantitative information of each dot was summarized in the volume object list. Unless otherwise stated, all the signal comparison of the images in this report was done based on the values of the adjusted volume with local background subtraction.

2.4 Results

2.4.1 Analysis of total RNA

Total RNA isolated by method adapted from Chirgwin *et al.* (1979) was analyzed. The absorbance of UV light at 260 nm and 280 nm were measured. A_{260}/A_{280} calculated was almost constant among the samples (1.43-1.53) (Table 2.2).

Table 2.2. **Spectrophotometric analysis of the isolated RNAs.**

Sample	A_{260}/A_{280}
Biochanin A sample set	
Untreated JCS cells	1.52
JCS cells treated with 0.25 % ethanol for 18 hours	1.43
JCS cells treated with 0.25 % ethanol for 46 hours	1.47
JCS cells treated with Biochanin A (50 μM) for 18 hours	1.50
JCS cells treated with Biochanin A (50 μM) for 46 hours	1.48
Midazolam sample set	
Untreated JCS cells	1.53
JCS cells treated with midazolam (10 μg/ml) for 18 hours	1.53
JCS cells treated with midazolam (10 μg/ml) for 48 hours	1.54

Absorbance at 260 nm and 280 nm was measured for each RNA sample. These RNAs were isolated from cells treated with either biochanin A (50 μM) or midazolam (10 μg/ml) for different period of time as indicated. A_{260}/A_{280} = ratio of absorbance at 260 nm to absorbance at 280 nm.

RT-PCR on each type of total RNAs was performed for the constitutively expressed gene GAPDH as described in section 2.3.2. The same reaction master mix was used for samples to be compared (i.e. those within the same sample set). The level of GAPDH gene expression analyzed by 20 cycles of PCR and gel electrophoresis was essentially the same for all the samples in either the biochanin A or the midazolam sample set (Figure 2.3).

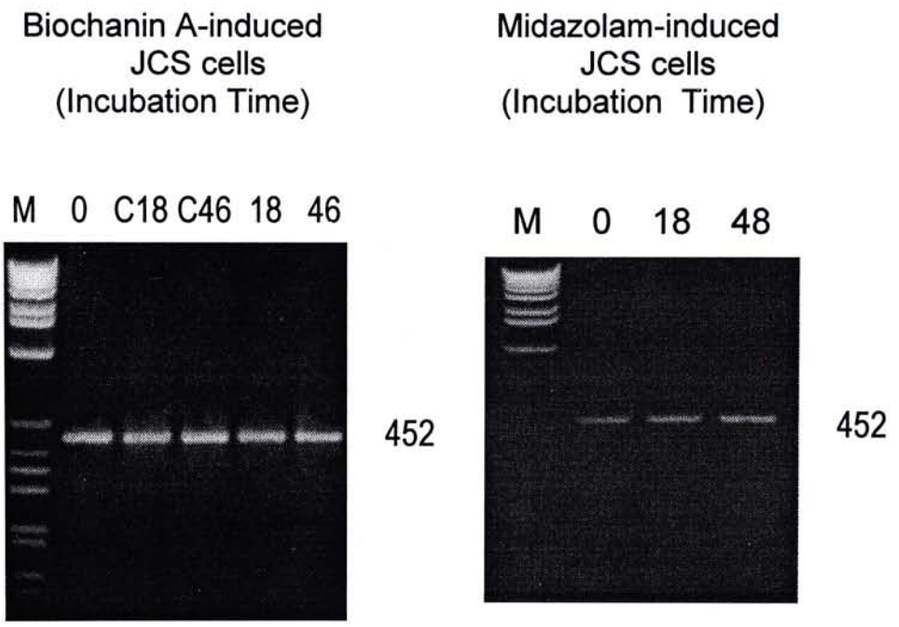


Figure 2.3. **Normalization of samples by GAPDH gene expression.** 20 cycles of RT-PCR using GAPDH gene specific primers were performed on RNA samples isolated form control JCS cells, JCS cells treated with 0.25 % ethanol for 18 and 46 hours (C18 and C46), JCS cells treated with biochanin A for 18 and 46 hours (18, 46) or midazolam for 18 and 48 hours (18, 48). 10 μl of the PCR products was electrophoresed on a 1.5 % TAE Synergel. The expected PCR product size was 452 bp. M = marker lane, 1Kb ladder.

2.4.2 mRNA phenotyping

Cycle titration together with either dot blot hybridization or Southern blot hybridization were used to characterize the expression of some chosen cytokines in a semi-quantitative approach (Dallman *et al.,* 1991; Dallman and Porter, 1992). The expression patterns of these cytokines during the induced myeloid cell differentiation fell into three categories :

1. No detectable or very low expression throughout the differentiation
2. Constitutive expression without significant change during the differentiation
3. Detectable change in expression level during the differentiation

Biochanin A-treated samples

In biochanin A-treated JCS cells, genes for LIF-R (transmembrane form) and LIF-R (soluble form) were undetectable using 35 and 50 cycles of RT-PCR and Southern blot hybridization and that for IL-4 was only barely detectable (after 50 cycles of RT-PCR and Southern blot hybridization) at 46 hours during the myeloid cell differentiation into monocytes (Figure 2.4). The expected PCR product size for IL-4, LIF-R (transmembrane form) and LIF-R (soluble form) were 422, 340 and 415 bp respectively as shown in the lanes of the positive controls.

In contrast to the genes described above, both types of IL-1 receptor, IL-1RtI and IL-1RtII (Figure 2.5), together with one form of the IL-3R β subunits AIC2B (Figure 2.8) showed constant levels of mRNA throughout the monocytic differentiation triggered by biochanin A as detected by RT-PCR and dot-blot hybridization.

Further, the expression of IL-1α and IL-1β was increased after JCS cells were treated with biochanin A for 46 hours (Figure 2.6), the time when the cells

demonstrated morphological changes (section 2.1.2). Unlike IL-1α, IL-1β showed also a basal level of expression in untreated JCS cells. In addition to IL-1, up-regulation of expression levels were observed in IL-3, TNF-α and LIF (Figure 2.7). Different from LIF expression, the level of IL-3 and TNF-α dropped at 18 hours followed by a rebound at a later time (46 hours). Moreover, this pattern of change was found in cells treated with solvent (0.25 % ethanol) alone though to a lesser extent. LIF expression, on the other hand was enhanced significantly at 46 hours after biochanin A treatment. The expression of LIF was unaffected by the solvent 0.25% ethanol (Figure 2.7).

Despite the constant expression of AIC2B of IL-3R (Figure 2.8), AIC2A, another form of β subunit was up-regulated in expression level after the cells were treated with biochanin A for 18 hours; the level dropped slightly at 46 hours after the treatment. Such change was not duplicated in JCS cells treated with solvent alone although a slight up-regulation of the gene was found after incubation with the solvent for 46 hours (Figure 2.8). Quite distinctive from the above observations, the expression of IL-3Rα subunits was up-regulated steadily from 18 hours to 46 hours of incubation with biochanin A while 0.25 % ethanol caused a down-regulation of the gene at 18 hours (Figure 2.8).

Finally, J11d, a differentiation marker was down-regulated steadily from 18 to 46 hour incubation in both the solvent and biochanin A-treated samples (Figure 2.7).

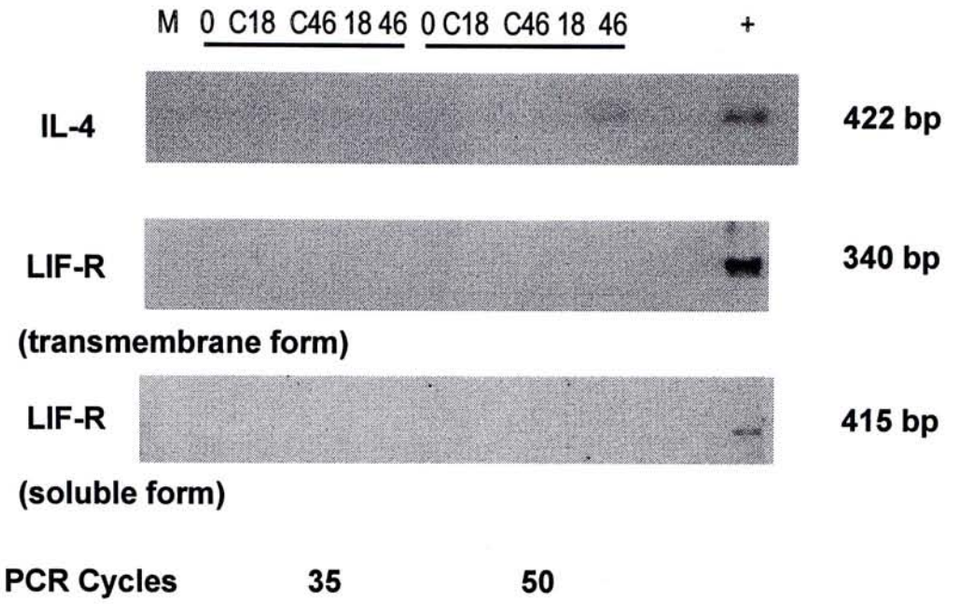


Figure 2.4. **Gene expression levels of IL-4, LIF-R (transmembrane form) and LIF-R (soluble form) during biochanin A-induced JCS cell differentiation.** RT-PCR was performed on RNA samples obtained from control JCS cells, JCS cells treated with 0.25 % ethanol for 18, 46 hours (C18, C46) or JCS cells treated with 50 μM biochanin A for 18, 46 hours (18 and 46) at the indicated no. of PCR cycles. PCR on DNA fragments having a partial sequence the same as the endogenous gene were included as a positive control (+). PCR products of these samples were electrophoresed on a 2 % TAE agarose gel. Southern blot hybridization was done using DIG-labeled internal probe specific for each gene. Chemiluminescent detection was done and the signals were developed on an X-ray film. The specific product sizes for the genes IL-4, LIF-R (transmembrane form) and LIF-R (soluble form) was 422, 340 and 415 bp respectively. M = position of the marker lane.

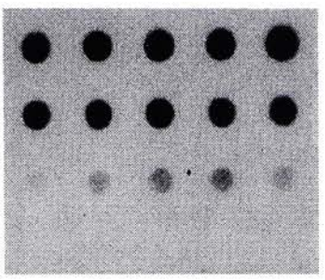
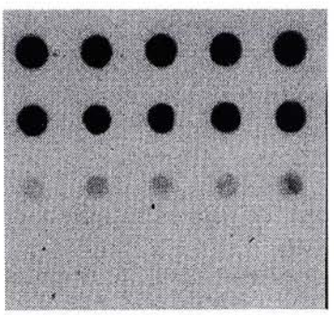
	PCR cycles	Time (hours)					Volume [ODXarea(mm^2)]				
		0	C18	C46	18	46	0	C18	C46	18	46
IL-1RtI	5+30						13.09	12.97	14.25	13.91	16.78
	5+25						11.33	10.87	12.16	11.18	11.07
	5+20						0.30	0.71	1.52	1.88	0.72
	5+15						-	-	-	-	-
IL-1RtII	5+35						12.72	12.51	12.36	12.51	11.77
	5+30						11.82	9.98	9.63	11.18	11.50
	5+25						0.34	0.81	0.61	0.71	1.45
	5+20						-	-	-	-	-

Figure 2.5. **Gene expression levels of type I and type II IL-1 receptors during biochanin A-induced JCS cell differentiation.** JCS cells were untreated (0), incubated with solvent 0.25 % ethanol for 18 (C18) and 46 (C46) hours or treated with 50 μM biochanin A for 18 (18) and 46 (46) hours. RT-PCR was performed on the RNAs isolated from these untreated and treated JCS cells at different cycle number as indicated. 10 μl of each type of RT-PCR products was dotted onto positively-charged membranes and hybridized to DIG-labeled internal probe specific for each gene. Chemiluminesent detection was performed and quantitative analysis of signal demonstrated by each dot was done by densitometric scanning. Each of the signals was converted into numerical value in terms of Volume [OD X area (mm^2)]. For PCR cycles, 5+N means 5 touchdown PCR cycles starting from (Tm-10) °C as annealing temperature at the first cycle of reaction which dropped 1 °C per each consecutive cycle for a total of 5 cycles followed by N cycles having an annealing temperature of (Tm-5) °C (see 3.1.3 for touchdown PCR).

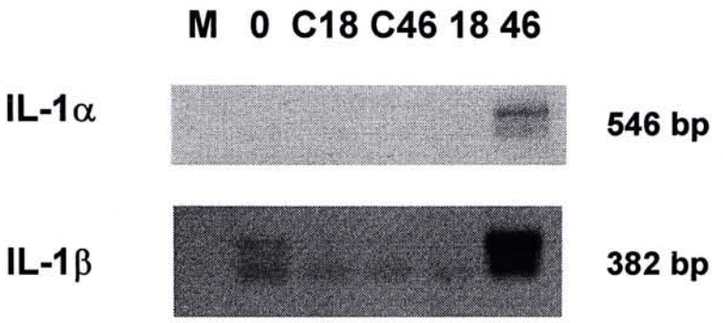


Figure 2.6. **Gene expression levels of IL-1α and IL-1β during biochanin A-induced JCS cell differentiation.** 35 cycles of RT-PCR using either IL-1α and IL-1β gene specific primers were performed on RNA samples isolated from control JCS cells, 0.25 % ethanol-treated cells for 18 and 46 hours (C18 and C46) or biochanin A-treated JCS cells for 18 and 46 hours (18 and 46). The PCR products were electrophoresed on a 2 % TAE agarose gel and Southern blot hybridization was done using DIG-labeled internal probes specific for the genes. Chemiluminescent detection was done and signals were developed on X-ray film. The expected product size for IL-1α and IL-1β were 546 and 382 bp respectively. M= position of the marker lane.

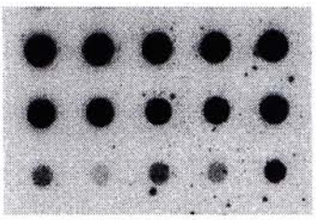
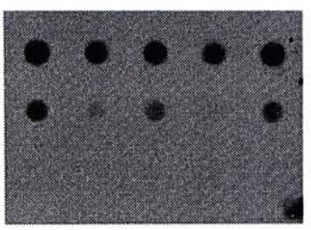
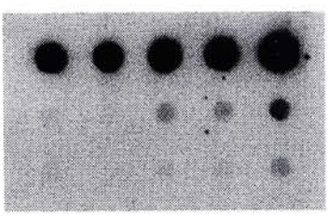
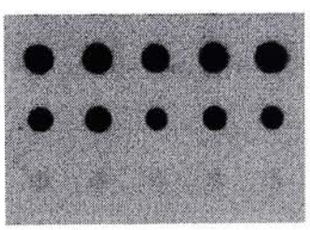
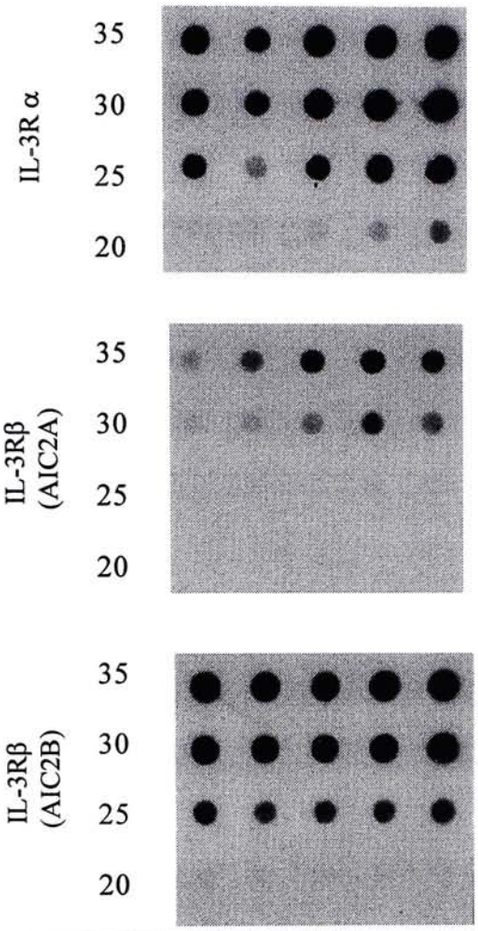
	PCR cycles	Time (hours)	Volume [ODXarea(mm^2)]				
		0 C18 C46 18 46	0	C18	C46	18	46
IL-3	30		12.51	13.00	13.62	13.50	13.46
	25		12.24	12.16	11.15	11.32	13.00
	20		2.24	0.54	2.11	1.44	5.90
TNF-α	35		7.61	5.74	6.28	5.24	7.20
	30		3.75	0.52	1.49	0.27	2.50
	25		-	-	-	-	-
LIF	30		20.09	19.45	24.40	24.02	29.05
	22		0.20	0.15	0.93	0.91	3.49
	20		0.10	0.03	0.18	0.12	0.81
J11d	30		14.12	15.05	13.29	14.42	15.36
	25		8.29	7.81	5.44	8.15	6.71
	20		0.34	0.25	0.15	0.27	0.11

Figure 2.7. **Gene expression levels of IL-3, TNF-α, LIF and J11d during biochanin A-induced JCS cell differentiation.** JCS cells were untreated (0), incubated with solvent control 0.25 % ethanol for 18 (C18) and 46 (C46) hours or treated with 50 μM biochanin A for 18 (18) and 46 (46) hours. RT-PCR were performed on the RNAs isolated from these untreated and treated JCS cells at different cycle numbers as indicated. 10 μl of each type of the RT-PCR products was dotted onto positively-charged membranes and hybridized to DIG-labeled internal probe specific for each gene. Chemiluminesent detection was performed and quantitative analysis of signal demonstrated by each dot was done by densitometric scanning. Each of the signals was converted into numerical values in terms of Volume [OD X area (mm^2)].



	PCR cycles	Time (hours) 0	C18	C46	18	46	Volume [ODXarea(mm^2)] 0	C18	C46	18	46
IL-3R α	35						11.87	8.17	13.60	14.07	12.88
	30						10.39	7.48	12.55	13.71	12.35
	25						7.13	1.10	6.70	10.70	10.83
	20						0.08	0.04	0.18	0.48	2.05
IL-3Rβ (AIC2A)	35						0.58	2.41	5.91	6.27	5.41
	30						0.21	0.39	1.23	3.78	2.06
	25						0	0.04	0.03	0.10	0.03
	20						-	-	-	-	-
IL-3Rβ (AIC2B)	35						13.30	12.93	12.45	13.61	1306
	30						12.54	11.55	11.50	12.05	12.51
	25						5.87	3.63	4.34	4.54	5.91
	20						0.09	0.01	0.04	0.03	0.04

Figure 2.8. **Gene expression levels of IL-3R subunits, IL-3Rα, IL-3Rβ (AIC2A) and IL-3Rβ (AIC2B) during biochanin A-induced JCS cell differentiation.** JCS cells were untreated (0), incubated with solvent control 0.25 % ethanol for 18 (C18) and 46 (C46) hours or treated with 50 μM biochanin A for 18 (18) and 46 (46) hours. RT-PCR were performed on the RNAs isolated from these untreated and treated JCS cells at different cycle number as indicated. 10 μl of each type of the RT-PCR products was dotted onto positively-charged membranes and hybridized to DIG-labeled internal probes specific for each gene. Chemiluminesent detection was performed and quantitative analysis of signal demonstrated by each dot was done by densitometric scanning. Each of the signals was converted into numerical values in terms of Volume [OD X area (mm^2)].

Midazolam-treated samples

Similar to biochanin A-induced JCS cells, the expressions of IL-4, LIF-R (transmembrane form) and LIF-R (soluble form) were undetectable in midazolam-treated samples. However, as opposed to biochanin A, midazolam did not enhance the expression of IL-1α which remained undetectable throughout the monocytic / granulocytic differentiation of JCS cells (Figure 2.9).

LIF, TNF-α (Figure 2.10) and IL-3Rα (Figure 2.13) had a constitutive expression during the midazolam-induced differentiation while IL-1β and IL-3 (Figure 2.11) showed a slight down-regulation of the expression levels. Transient down-regulation of type II IL-1 receptor was observed at 18 hours while type I receptor was up-regulated in expression at 48 hours after induction by midazolam (Figure 2.12).

Also, a significant up-regulation of β subunits of IL-3 receptor was found (Figure 2.13). Both AIC2A and AIC2B were elevated slightly in expression after JCS cells were treated with midazolam for 48 hours, in contrast to the unchanged expression illustrated by the α subunit.

The expression of J11d was also studied; the marker had steady increase in expression during the midazolam-induced differentiation (Figure 2.11) as opposed to that during the biochanin A-induced differentiation.

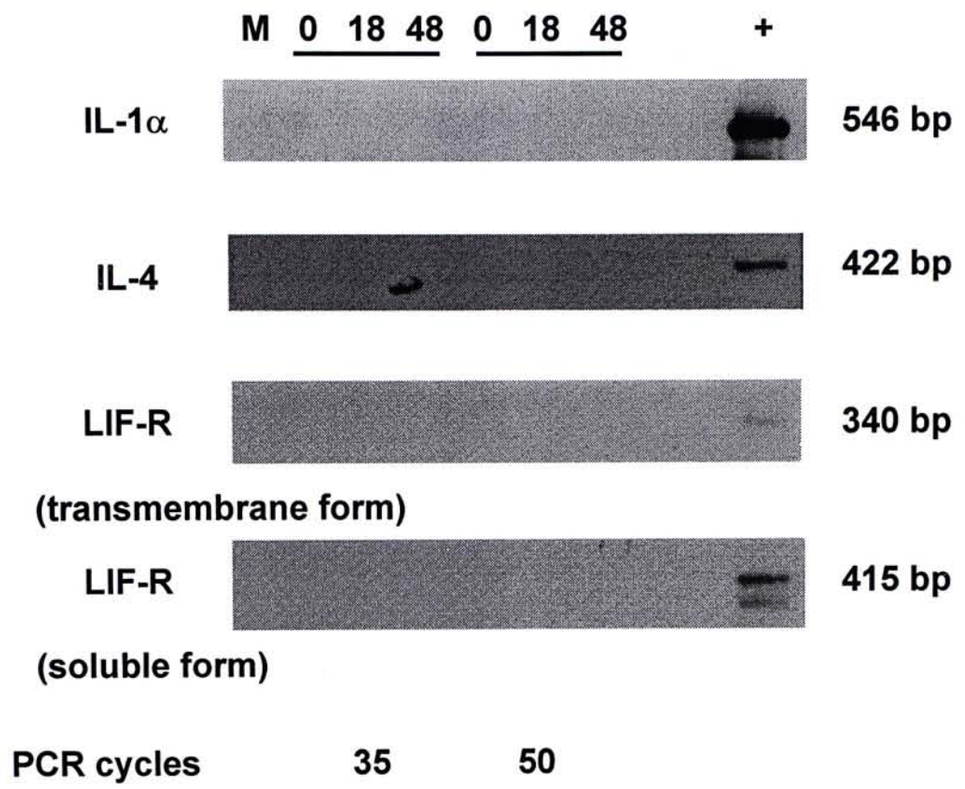


Figure 2.9. **Gene expression levels of IL-1α, IL-4, LIF-R (transmembrane form) and LIF-R (soluble form) during midazolam-induced JCS cell differentiation.** 35 and 50 cycles of RT-PCR were performed on the RNAs isolated from untreated JCS cells, JCS cells treated with 10 μg/ml midazolam for 18 and 48 hours. PCR done on DNA fragments having a partial sequence of the endogenous gene were included as a positive control (+). All these PCR products were electrophoresed on a 2 % TAE agarose gel. Southern blot hybridization was done using DIG-labeled internal probe specific for each gene. Chemiluminescent detection was done and the signal were developed on an X-ray film. The specific product sizes for the genes IL-1α, IL-4, LIF-R (transmembrane form) and LIF-R (soluble form) were 546, 422, 340 and 415 bp respectively. M = position of the marker lane.

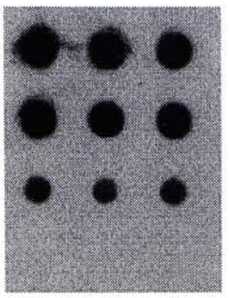
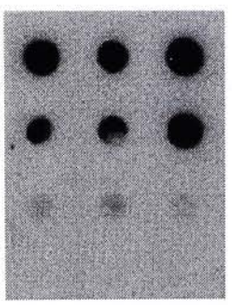
	PCR cycles	Time (hours)			Volume [OD x area(mm^2)]		
		0	18	48	0	18	48
LIF	40				13.98	13.73	12.15
	35				10.26	10.61	10.38
	30				4.12	3.13	4.05
	25				-	-	-
TNFα	35				12.39	9.21	14.10
	30				4.75	5.43	9.21
	25				0.45	0.48	0.33
	20				-	-	-

Figure 2.10. **Gene expression levels of LIF and TNF-α during midazolam-induced JCS cell differentiation.** RT-PCR were performed on control JCS cells, JCS cells treated with 10 μg/ml midazolam for 18 and 48 hours at the PCR cycles indicated. 10 μl of each type of the RT-PCR products was dotted onto positively-charged membranes and hybridized to DIG-labeled internal probe specific for each gene. Chemiluminesent detection was performed and quantitative analysis of signal demonstrated by each dot was done by densitometric scanning. Each of the signals was converted into numerical values in terms of Volume [OD X area (mm^2)].

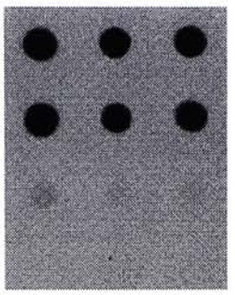
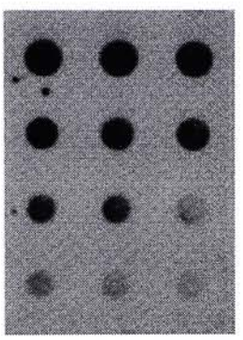
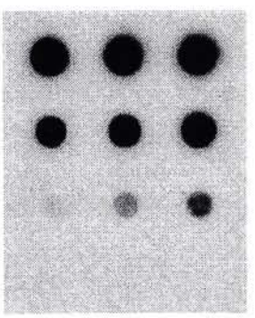
	PCR cycles	Time (hours)			Volume [OD x area(mm^2)]		
		0	18	48	0	18	48
Il-1β	35				6.85	5.97	5.47
	30				6.96	5.70	5.84
	25				0.47	0.06	0.17
	20				-	-	-
IL-3	22				12.39	10.85	10.28
	20				7.48	7.68	7.26
	18				3.99	3.91	1.09
	16				1.20	0.73	0.53
J11d	35				16.11	15.95	16.83
	30				10.37	11.23	12.86
	25				0.24	1.06	2.71
	20				-	-	-

Figure 2.11. **Gene expression levels of IL-1β, IL-3 and J11d during midazolam-induced JCS cell differentiation.** RT-PCR were performed on control JCS cells, JCS cells treated with 10 μg/ml midazolam for 18 and 48 hours at the PCR cycles indicated. 10 μl of each type of the RT-PCR products was dotted onto positively-charged membranes and hybridized to DIG-labeled internal probes specific for each gene. Chemiluminesent detection was performed and quantitative analysis of signal demonstrated by each dot was done by densitometric scanning. Each of the signals was converted into numerical values in terms of Volume [OD X area (mm^2)].

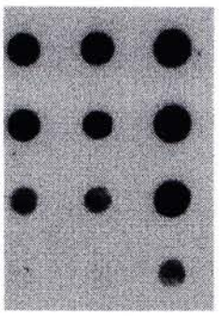
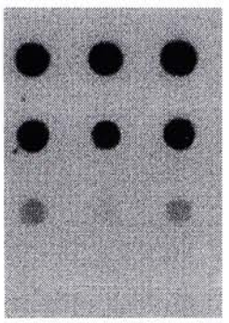
	PCR cycles	Time (hours)			Volume [OD x area(mm^2)]		
		0	18	48	0	18	48
IL-1RtI	5+35				10.89	11.76	15.34
	5+30				10.83	8.17	15.30
	5+25				4.35	4.19	14.32
	5+20				0.22	0.21	3.30
IL-1RtII	5+35				11.14	11.27	12.62
	5+30				9.70	5.92	9.80
	5+25				1.03	0.14	0.74
	5+20				-	-	-

Figure 2.12. **Gene expression levels of type I (IL-1RtI) and type II (IL-1RtII) IL-1 receptors during midazolam-induced JCS cell differentiation.** RT-PCR were performed on control JCS cells, JCS cells treated with 10 μg/ml midazolam for 18 and 48 hours at the PCR cycles indicated. 10 μl of each type of the RT-PCR products was dotted onto positively-charged membranes and hybridized to DIG-labeled internal probes specific for each gene. Chemiluminesent detection was performed and quantitative analysis of signal demonstrated by each dot was done by densitometric scanning. Each of the signals was converted into numerical values in terms of Volume [OD X area (mm^2)]. For PCR cycles, 5+N means 5 touchdown PCR cycles starting from (Tm-10) °C as annealing temperature at the first cycle of reaction which dropped 1 °C per each consecutive cycle for a total of 5 cycles followed by N cycles having an annealing temperature of (Tm-5) °C (see section 3.1.3 for touchdown PCR).

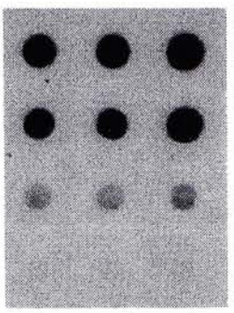
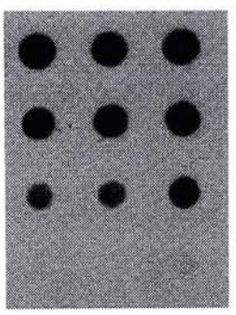
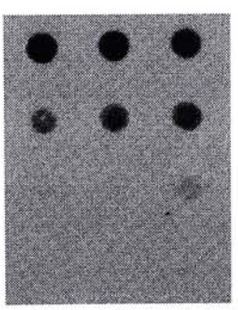
	PCR cycles	Time (hours)			Volume [OD x area(mm^2)]		
		0	18	48	0	18	48
Il-3Rα	30				11.08	11.11	14.02
	25				9.23	9.69	12.65
	20				0.68	0.62	1.33
	15				0.07	0.01	0.10
IL-3Rβ (AIC2A)	35				8.56	7.92	8.26
	30				8.26	8.45	8.50
	25				3.28	3.71	6.47
	20				0.03	0.06	0.19
IL-3Rβ (AIC2B)	35				6.24	5.72	5.26
	30				2.20	3.40	3.77
	25				0.03	0.02	0.52
	20				-	-	-

Figure 2.13. **Gene expression levels of IL-3R, α subunits (IL-3Rα), β subunits (AIC2A) and (AIC2B) during midazolam-induced JCS cell differentiation.** RT-PCR were performed on control JCS cells, JCS cells treated with 10 μg/ml midazolam for 18 and 48 hours at the PCR cycles indicated. 10 μl of each type of the RT-PCR products was dotted onto positively-charged membranes and hybridized to DIG-labeled internal probes specific for each gene. Chemiluminesent detection was performed and quantitative analysis of signal demonstrated by each dot was done by densitometric scanning. Each of the signals was converted into numerical values in terms of Volume [OD X area (mm^2)].

2.4.3 Summary of mRNA phenotyping results

Comparing biochanin A-and midazolam-induced JCS cells (Table 2.3), there were genes that showed the same types of expression pattern. As mentioned, IL-4 and both forms of LIF receptor were not expressed or expressed at a very low level after either kind of treatment. However, there were more genes that were affected by the two compounds differently. While IL-1α was up-regulated in biochanin A-induced JCS cells at 46 hours, the gene was not expressed throughout the treatment of midazolam in JCS cells. For IL-1β, the expression pattern went in an opposite direction. While IL-1β gene expression was up-regulated by biochanin A, the gene was down-regulated by midazolam. The expression level of IL-1 receptor was also different under different kinds of induction. Both types of IL-1 receptors had constant level of expression despite biochanin A treatment. In contrast, the expression of type I receptor was up-regulated while that of type II receptor was transiently down-regulated at 18 hours. Discrepancies of mRNA levels of IL-3 and its receptor between biochanin A-induced and midazolam-induced JCS cells were also found. IL-3 was up-regulated in expression during biochanin A-stimulated differentiation but down-regulated during midazolam-mediated differentiation. Although α subunit of IL-3 receptor was unchanged in expression during midazolam-induced differentiation, the gene was increased in expression induced by the combined action of biochanin A and 0.25 % ethanol. By contrast, both forms of β subunits were up-regulated in midazolam-treated cells but unchanged in biochanin A-treated cells. With respect to TNF-α, the gene was up-regulated by biochanin A plus its solvent but remained unchanged in midazolam-induced cells. While LIF gene expression was up-regulated by biochanin A, the expression level remained unchanged after midazolam treatment. As for J11d, the expression was decreased in biochanin A-treated JCS cells but enhanced in midazolam-treated cells.

Table 2.3. **Expression profiles of the cytokines, cytokine receptors and marker gene studied.**

Genes	Biochanin A sample set					Midazolam sample set		
	0.25 % ethanol			Biochanin A (50 μM)		Midazolam (10 μg/ml)		
	0 hr	18 hr	46 hr	18 hr	46 hr	0 hr	18 hr	48 hr
IL-1α	-	-	-	-	+	-	-	-
IL-1β	+	+	+	+	++	++	+	+
IL-3	++	+	++	+	+++	++	++	+
IL-4	-	-	-	-	-	-	-	-
TNF-α	++	+	++	+	++	+	+	+
LIF	+	+	++	++	+++	+	+	+
IL-1RtI	+	+	+	+	+	++	++	+++
IL-1RtII	+	+	+	+	+	++	+	++
IL-3Rβ	+	+	+	+	+	+	+	++
IL-3Rα	++	+	++	++	+++	+	+	+
LIF-R	-	-	-	-	-	-	-	-
J11d	++	++	+	++	+	+	+	++

'+' indicates expression. '-' indicates no expression. The increasing or decreasing number of '+" indicates the relative increase/decrease in expression of the same gene only, there was no comparison of gene expression among different genes.

2.5 Discussion

2.5.1 mRNA phenotyping

Numerous cytokines have been implicated in myeloid cell differentiation. Both the direct and indirect effects of exogenously added cytokines and endogenously produced cytokines have been studied. (Hass *et al.,* 1991; Jacobsen *et al.,* 1994; Lotem and Sachs, 1989).

Following previous studies, we continued to examine the expression of cytokines in terms of mRNA levels in JCS cells and evaluate their possible involvements in induced myeloid cell differentiation.

The exogenouly added TNF-α has been shown to suppress proliferation of early hematopoietic progenitors of bone marrow cells and the autocrine effect of TNF-α allowed the cells to undergo terminal differentiation (macrophage) (Witsell and Schook, 1992). Exogenously added TNF-α was shown to be a potent inducer of JCS cells differentiating into monocytes previously (Mak *et al.,* 1993). The cytokine itself stimulated further elevated expression of endogneous TNF-α (Chan *et al.,* 1995). In the current studies, we are interested to know whether such induction is specific for the cytokine TNF-α stimulation or for the monocytic differentiation of the cells. From the expression profile analyzed by RT-PCR, it appeared that such TNF-α induction was unique in differentiation stimulated by TNF-α. There was no observable change in expression associated with the monocytic/granulocytic differentiation stimulated by midazolam (Figure 2.10). Although TNF-α was slightly up-regulated by the combined effect of biochanin A and 0.25 % ethanol, the change did not seem to be correlated with the differentiation commitment triggered by biochanin A (Figure 2.7). This is because the same pattern of change occurred in JCS cells treated with 0.25 % ethanol alone where no phenotypic changes of differentiation were observed. Despite the degree of change was higher in the experimental samples, the increase occurred only at 46 hours which would be too late for influencing the differentiation program.

The increase in TNF-α gene expression in biochanin A-induced but not midazolam-induced sample may be related to the different effect of the two inducers. Alternatively, it is possible that TNF-α production is required in greater demand for cells committed entirely to monocytes (biochanin A induced cells) than cells committed to produce both granulocytes and monocytes (midazolam-induced cells).

Two other important inducers of JCS monocytic differentiation, namely IL-1α and IL-1β, were also induced by TNF-α. In addition, the differentiation-inducing action of IL-1α was mediated through the action of IL-1β (Chan *et al.,* 1995). From the present studies, IL-1α was not expressed during the monocytic/granulocytic differentiation triggered by midazolam (Figure 2.9) while IL-1β showed a little down-regulation (Figure 2.11). However, it is doubtful to see such slight change of IL-1β expression would have any particular effect in determining the monocytic/granulocytic differentiation. Elevated mRNA levels of both IL-1α and IL-1β were shown in biochanin A-induced JCS cells. Nevertheless, the change did not occur until after 46 hour of induction. Since IL-1 is readily produced by macrophage, increased production of IL-1 at a late time point (46 hours) triggered by biochanin A may be correlated with cell maturation and function. This induced IL-1 production is biochanin A specific as no expression of IL-1 and decreasing mRNA level of IL-1β were observed in midazolam-induced cell population, having monocytic cells (in addition to granulocytic cells).

The expression of IL-1 receptors (both types) were unchanged (Figure 2.5) during biochanin A-induced cell differentiation and suggested that the response of JCS cells to IL-1 was not changed throughout the differentiation. On the other hand, the response of midazolam-induced JCS cells to IL-1 would have been increased slightly at 18 hours as the expression of decoy receptor IL-RtII (reviewed in Dinarello, 1994) was decreased transiently at 18 hours (Figure 2.12) The response to IL-1 may also be increased significantly at 46 hours as the expression of the signaling receptor IL-1RtI was increased markedly at 46 hours (Figure 2.12). Since the increase in signaling receptor (IL-1RtI) and decrease in non-signaling receptor (IL-1RtII) did not accompany with an increase in IL-1 production in midazolam-treated cells, the

differentiating action of IL-1 on monocytic/granulocytic JCS cell differentiation, if any (at 18 hours), could possibly be effected only through exogenous source of IL-1. The increase in expression of type I receptors at 46 hours may be related to the function of neutrophils in the cell population of midazolam-induced JCS cells. Neutrophils are frequently recruited to the site of inflammations by IL-1.

TNF-α and IL-1 have been suggested to have very similar biological functions in many aspects and both of them stimulated monocytic differentiation of JCS cells (Chan *et al.,* 1995). While TNF-α and IL-1β probably signaled the cells in two independent pathway, IL-1α effect its action through IL-1β (Chan *et al.,* 1995). As TNF-α caused increased expression of both forms of IL-1, interacting cytokine actions in JCS monocytic differentiation were suggested. In contrast, the current studies indicated a third unique pathway of monocytic differentiation (initiated by biochanin A) that involved neither TNF-α nor IL-1. Similarly, both TNF-α and IL-1 played no part in midazolam triggered monocytic/granulocytic differentiation but this does not preclude the involvement of these cytokines in granulocytic differentiation of JCS cells. In short, the present data and previous report suggested that TNF-α and IL-1 mRNA induction was unique in TNF-α triggered pathway of monocytic differentiation.

IL-4 is a pleiotropic cytokine having various effect on B cells, T cells, monocytes, endothelial cells and fibroblasts. It is involved in the expansion and recruitment of early myeloid progenitors together with other cytokines (IFN-γ, IL-1 and IL-3) (Snoeck *et al.,* 1996) and modulates the growth of hematopoietic progenitor cells (Rennick *et al.,* 1987). During terminal differentiation, the increased level of IL-4 (relative to IFN-γ) preferentially gives rise to granulocytes rather than monocytes (Snoeck *et al.,* 1996). Based on the previous published work, IL-4 and TNF-α have synergistic effect in inducing JCS cells to undergo monocytic differentiation (Leung *et al.,* 1994). In addition, IL-4 was enhanced in expression during the TNF-α–induced differentiation of JCS cells after 18 hour of induction (Chan *et al.,* 1995). By contrast, IL-4 was not expressed during either the biochanin A-or midazolam-stimulated

differentiation in the current investigation (Figure 2.4, 2.9) and thus excluded the effect of IL-4 on JCS cell differentiation induced by the two compounds.

Autocrine loop of IL-3 production is a characteristic of this cell model. Its parent cell line WEHI-3B has an intracisternal A particle (IAP) inserted with its 5' long terminal repeat (LTR) close to the promoter region of the IL-3 gene leading to a continuous production of the growth factor. Such phenomenon has been shown to contribute to the leukemic phenotype of the cells (Perkins *et al.,* 1990). During the midazolam-induced monocytic/granulocytic differentiation of JCS cells, a slight down-regulation of gene expression level was observed (Figure 2.11). This observation may be correlated with the anti-proliferative effect of midazolam. It has been reported that several peripheral benzodiazepine receptor-specific ligands which suppressed the induced proliferation of human mononuclear cells were able to inhibit interleukin-3-like activity secretion (Besseler *et al.,* 1992), although such activity of midazolam has not been studied.

The α subunit of the IL-3 receptors determines the specificity was unresponsive to midazolam but the β subunit AIC2A which binds weakly to IL-3 and AIC2B which causes signal transduction and complexes with other cytokine receptor subunits were slightly up-regulated at 48 hours (Figure 2.13). Whether such kind of receptor expression suggested an increasing demand for IL-3 on maturation, or associated with the function of other cytokine receptors (GM-CSFR and IL-5R) which share AIC2B with IL-3 receptor was unknown. Likewise, the increase in IL-3 gene expression did not seem to be correlated with the observed differentiation induced by biochanin A (Figure 2.7). Instead of differentiation-related, the increased level of IL-3 expression induced by both 0.25 % ethanol (solvent control) and biochanin A may reflect an increase demand for IL-3 as a survival factor. Unlike the midazolam treatment, biochanin A did not alter the expression of the β subunits of IL-3R. Instead, elevated expression was found in the α subunit. This increase in expression of IL-3Rα may help in competing for AIC2B (β subunit) with GM-CSFRα or IL-5Rα (Nicola and Metcalf, 1991). Hence, the response of JCS cells to IL-5 or GM-CSF would be significantly decreased (Walker *et al.,* 1985). It was also found that 0.25 %

ethanol suppressed the expression of IL-3Rα but no significant differentiation nor inhibited proliferation was observed. This result is worth noting as weak differentiation-inducing effect of ethanol at higher concentration has been observed (1%) (personal communication with Dr. N.K.Mak, unpublished data). It is suggestive that ethanol-induced differentiation may effect through inhibited proliferation of JCS cells caused by a decreased response to IL-3.

LIF, leukemia inhibitory factor, as the name implied was first discovered to cause monocytic differentiation of M1 leukemia cell line. However, both the differentiation-inducing effect of LIF and its expression in JCS cells have not been investigated. In the present study, LIF gene expression was detected in untreated JCS cells and was increased as the cells differentiated along the monocytic lineage induced by biochanin A (Figure 2.7). Howver, no change in LIF gene expression was observed during the monocytic/granulocytic differentiation caused by midazolam (Figure 2.10). On the other hand, both forms of LIF-R were not expressed in either the treated or untreated sample. This ruled out the possible involvement of LIF in affecting the differentiation of JCS cells despite an elevated level of LIF expression in biochanin A-treated cells. The unresponsiveness of cells to exogenous LIF to differentiate has also been shown in the related cell line WEHI-3B(D^+) (Hilton *et al.*, 1988).

The increased LIF expression at 46 hours during the monocytic differentiation of JCS cells induced by biochanin A (Figure 2.7) may correlate with the function of the mature cells. Cells of the myelomonocytic lineages are producers of LIF (Anegon *et al.*, 1990) which in turn mediates a lot of diverse biological functions (reviewed in Hilton, 1992). Dissimilar from other's work where LIF-R was found on monocytes, we cannot detected the receptors under the current system. This probably suggests defective expression of LIF-R in this cell line or such receptors are expressed at a very late stage of maturation (after 2 days of induction).

The marker gene J11d expression was also studied. J11d antigen is thought to be attached to a glycosylphosphatidyl-inositol membrane anchor. The antigen is

expressed in neutrophils but is absent in mature macrophages. In a report on M1 cells, both the J11d mRNA level and antigen expression were significantly decreased during the LIF-induced monocytic differentiation (Fung *et al.,* 1992) suggesting that J11d may be used as a lineage-specific (monocytic) and differentiation-related marker. Increase in mRNA levels of J11d in midazolam-induced JCS cells committed to give granulocytes (in addition to monocytes) further supports such view. However, the differentiation-associated decrease in expression of J11d induced by the combined action of biochanin A and its solvent is arguable since the degree of down-regulation of J11d expression was essentially the same as that in the solvent control sample where no differentiation of the cells was observed.

After examining the mRNA phenotypings, we concluded that the cytokines studied, namely IL-1, IL-3, IL-4, LIF and TNF-α, did not play an important role in signaling the monocytic and monocytic/granulocytic differentiation induced by biochanin A and midazolam respectively. Nevertheless, the results demonstrated that gene expression could be altered by inducers in JCS cells; some of these changes should be accounted for the differentiation commitment. Also, different gene expression patterns were triggered by different inducers driving cells to different lineages; some of these differences should be decisive in lineage specification. These observations led us to initiate the search for differentially expressed genes during the induced differentiation of JCS cells in Chapter 3 and 4.

2.5.2 Cytokine gene regulation

Using the current method in studying cytokine regulation, we should be careful about the interpretation because studying mRNA level of the genes does not give insight into how the genes are regulated. For instance, an increase in cytokine mRNA levels can be due to an increase in transcription rate or stability of the pre-existing mRNAs. The induced increase of some cytokine mRNAs like that of GM-CSF has been suggested not only due to an enhanced level of transcription but also a significant increase in the stability of the mRNA (reviewed in Greenberg and Belasco, 1993). Further, we should not overlook the control of cytokine genes other than that at the transcriptional level. Cytokine gene regulation can also be done at translation level, through glycosylation (Opdenakker *et al.,* 1995), precursor processing (e.g. IL-1 and TNF-α) (Carinci *et al.,* 1992), sequestration by soluble factors/receptors (e.g. TNF and IL-1) or extracellular matirx (e.g IL-1 and LIF), receptor modulation and receptor antagonist (IL-1).

As differentiation needs a very precise control, both initiation and shut-off of cytokine genes are important. Many cytokine genes have been shown to have an AU-rich destablizing elements at the 3' untranslated region. These genes are induced within minutes and are rapidly degraded having half-lives of minutes to a few hours as demonstrated in activated mononuclear cells (reviewed in Turner and Feldmann, 1990). LIF which has been found important in hematopoiesis was also transiently regulated in murine bone marrrow stromal cells. With AU-rich motifs at the 3' untranslated region, the LIF mRNA has a short half-life of 30 minutes (Derigs and Boswell, 1993). In contrast to the expression of LIF and that of IL-1β in activated mononuclear cells, the mRNA expression profile of IL-1β in differentiating U937 cells was different. The gene was induced within 4 hours after treatment of PMA and the elevated mRNA level was maintained from 8 hours to 3 days after PMA stimulation (Nishida *et al.,* 1988). High levels of mRNA transcripts of endogenous TNF-α, IL-1α and IL-1β were also maintained at 18 hours after induction by exogenous TNF-α in JCS cells (Chan *et al.,* 1995). These findings demonstrated that

expression profile of the same gene in myeloid cell differentiation can be different from that in other processes. Moreover, different genes that are important to cell differentiation or hematopoiesis may also have different degree of mRNA stability and expression time range.

In short, the above discussion does not invalidate the conclusion that biochanin A or midazolam-induced JCS cells followed a different differentiation pathway from that of the TNF-α–induced JCS cells. The only problem is that we cannot exclude the involvement of the examined cytokines during early hours of the differentiation. As many genes like proto-oncogenes and transcription factors other than cytokines showed transient expression at early hours, it is necessary for us to extend the range of our time course study in the following studies (Chapter 3 and 4). Hence, we will have a more conclusive view of the overall effect of both the transcriptional activation rate and transcript degradation of a particular gene.

2.5.3 mRNA quantitation using the current method

The current method is good for detecting dramatic changes of mRNA expression among samples. However, small changes or marginal changes may not be readily shown using five PCR cycle intervals. As a result, careful titration of different cycle numbers within the exponential phase and the cycle interval must be empirically determined for each gene. In this report, Southern blotting and dot-blotting were employed in different situation. Southern blotting is used for genes that have shown no or very low expression. The specificity of the primers giving products of expected size was demonstrated in the lane of positive control even though no signal or weak signal is detected in the experimental lanes (Figure 2.4, 2.9). On the other hand, PCR products might be analyzed by dot blot hybridization if amplification conditions had been optimized to yield single product (that hybridized to the internal probe). Obtaining single product is critical as conditions in the amplification may become limiting before a maximal amount of specific product is generated. Hence, the comparison of mRNA levels among samples may become invalid. Designing primers spanning the splice junctions in the RNA may help in avoiding undesirable products amplified from the genomic DNA. More importantly, amplification conditions like temperature, $MgCl_2$ concentration should be optimized to eliminate non-specific products. The touchdown PCR protocol employed in this report was empirically tested for the primer used; it is very effective for obtaining single PCR products.

With reference to the non-radioactive detection methods, high background and non-specificity at region of labeled nucleotides have been claimed (Hopman *et al.*, 1995). However, these problems have not been encountered in our protocol, probably because the labeling region is kept to a minimum (end-labeling). Different from radioactive detection, shortened exposure time to the X-ray film may not help in improving the non-linearity of X-ray film in the current system. As a result, the experiment may need to be repeated with lower cycle numbers to show the changes.

Although standards for quantitation of target DNA was omitted in the current method, an external endogenous gene is used to ensure the conversion of RNAs to cDNA and PCR amplification are equivalent among samples. β–actin, β–microglobulin, aldolase A, elongation factor EF-1α, GAPDH (glyceraldehyde-3-phosphate dehydrogenase, HPRT (hypoxanthine phosphoriboxyltransferase), histone H3.3 or ribosomal protein L19 appeared to be good choices as controls. However, the expression of these genes may be affected by experimental or physiological conditions (Bereta and Bereta, 1995; Mansur *et al.,* 1993); and the presence of psuedogenes may also influence the PCR amplification (Dirnhofer *et al.,* 1995). In addition, it seems that no single control gene can be completely free of these problems. Luckily, we have not observed such problems of GAPDH in our cell system; the gene showed unchanged mRNA levels at low PCR cycle number (20) in untreated as well as biochanin A- or midazolam-treated JCS cells.

In conclusion, the current method is able to compare the mRNA levels with notable change among different samples and is fast, simple, sensitive and readily applied to different genes with minimal optimization of conditions (temperature, touchdown PCR). Moreover, the steps involving manipulation of RNAs were minimized.

Chapter Three Identification and Isolation of Genes that are Differentially Expressed During Midazolam-induced JCS Cell Differentiation

3.1 Introduction

3.1.1 Methods for studying differentially expressed genes

To study differentially expressed genes in cells under different conditions, an efficient method is very crucial. Methods depending on biological assay of phenotype or gene function would be very useful but these assays are difficult to devise in most of the cases. Thus, a universal method to all genes meeting the following criteria is much more desirable: (adapted from Liang *et al.*, 1994)

1. It should allow comparison of mRNAs from different sources or different time point of treatment at the same time.
2. The mRNAs or cDNAs, in full-length or in partial length, that have been isolated could be used as a starting point to trace the corresponding genes.
3. All or most of the mRNAs in the cell could be visualized.
4. The method should be highly reproducible.
5. It should be sensitive, fast and easy.

There are a number of approaches and methods that suit some or most of the above criteria. Notably, differential screening strategies, subtractive hybridization and its derived strategies, as well as RNA fingerprinting and differential display by arbitrarily-primed PCR have been proved to be successful in isolating important genes.

Differential screening involves the construction of a cDNA library from source A whose gene expression is of particular interest. This library is then screened with

labeled cDNA from a second source, B. The non-hybridizing clones are then further analyzed by a suitable method like northern blotting. Only those clones that reproducibly give greater hybridization to RNA from source A than source B are further sequenced and characterized. Alternatively, a cDNA library made from source A were screened with labeled cDNA from both sources in parallel. Only clones that hybridized with probe A but not probe B were isolated. Differential screening suffers from two major drawbacks. The unmanipulated cDNA probes exhibit significant background hybridization and can only be used to isolate very abundantly expressed sequences (at least 0.1 % of messages) that can be identified above background hybridization. Second, large amounts of RNA from both sources are needed. Recently, the differential screening procedures are incorporated with the PCR techniques to reduce the required amount of starting materials without affecting the relative abundance of individual mRNA species. In some protocols, the cDNA from source A is amplified before it is cloned into the vectors (Smith and Gridley, 1992), while in others both cDNA populations designated to make probes and libraries were amplified by PCR (Brunet *et al.,* 1991) before screening.

To improve the sensitivity and to reduce the background, subtractive hybridization may be a choice. The method was designed to enrich moderate (0.01-0.1 % messages) to low abundance (less than 0.01 % of messages) genes. Slightly different from differential screening and other methods, subtractive hybridization technology aims at removing sequences common to both samples and enriching for clones that represent differentially expressed genes. To perform a typical subtractive hybridization, a population of molecules containing the desired message (target), say source A is hybridized with a 10- to 30-fold excess of molecules having messages to be removed (driver), source B. The unhybridized or enriched target molecules (A) are then separated from the hybrid molecules and the excess drivers. The enriched target molecules (A) can then be used to make either subtractive probes or a subtractive library. Subtractive probes enriched with target sequences can be used to screen a library made from the desired sequences (A) (Travis and Sutcliffe, 1988). Alternatively, if a subtractive library is made, desired sequences can be identified by screening in duplicate by subtractive probes and probes from source B. Clones

hybridized only with the subtractive probes are picked for further investigation (Hajnal *et al.,* 1994). There are many variations in subtractive methods in terms of starting materials for subtraction. Originally, subtraction was done between cDNA target sequence and poly(A)$^+$ driver sequences (Sargent, 1987; Sargent and Dawid, 1983). Then, subtractions were done between single-stranded DNA (ssDNA) from two libraries (Duguid *et al.,* 1988; Rubenstein *et al.,* 1990), ssDNA from one library and cRNA from the other library (Tanaka *et al.,* 1994; Li *et al.,* 1994a), ssDNA from one library and isolated cDNA form another source (Rivolta and Wilcox, 1995) or cRNA from one library and cDNA from another library (reviewed in Lawler *et al.,* 1993). Again, PCR technology has been introduced and combined with subtractive hybridization to improve the method. Frequently, PCR amplification was done on the subtractive cDNA population (Schweinfest *et al.,* 1993; Timblin *et al.,* 1990). PCR may also be performed to amplify both the driver and the tester before and after subtraction (Duguid and Dinauer, 1989). Subtractive protocol also deviates from each other by the method of separating (removing) the unhybridized species from the hybridized ones. Hydroxylapatite column chromatography (HAP) (Sargent and Dawid, 1983), phenol extraction of biotin-streptavidin-crosslinked polynucleotides (Duguid and Dinauer, 1989), magnetic beads (Aasheim *et al.,* 1994; Lonneborg *et al.,* 1995; Lopez-Fernandez and del Mazo, 1993) and enzymatic degradation (Rivolta and Wilcox, 1995; Zeng *et al.,* 1994) were employed by different authors. Alternatively, PCR techniques can be used to amplify preferentially the enriched target sequences in an exponential ratio but not the excess driver sequence or the hybrid sequence (Representational difference analysis or RDA) to circumvent the loss during separation procedures (Hubank and Schatz, 1994; Tsuchiya *et al.,* 1994).

Despite the improvement of the subtractive methods since its first use in mid-1970s, the technical aspect of the method is still very challenging. Subtractive methods have a high sensitivity and are good for rare messages. However, they are time-consuming, laborious and technically difficult. Moreover, they allow only one-way comparison. It is also impossible to compare several samples at the same time.

3.1.2 RNA fingerprinting by arbitrarily-primed PCR (RAP-PCR) and differential display (DDRT-PCR)

Two very similar protocols were devised in 1992, namely, RNA fingerprinting by arbitrarily-primed PCR (RAP-PCR) by Welsh *et al.* (1992) and differential display (DDRT-PCR) by Liang and Pardee (1992). As the names implied, both methods make use of PCR technique. They are semi-quantitative and allow multiple samples to be analyzed simultaneously. These protocols have found applications in many research areas including embryogenesis (Zimmermann and Schultz, 1994), cancers (Wu *et al.,* 1995), heart diseases (Utans *et al.,* 1994), diabetes (Nishio *et al.,* 1994), brain and neurology (Dalal *et al.,* 1994; Livesey and Hunt, 1996), growth factor stimulation and inhibition (Ralph *et al.,* 1993), plant systems (Momiyama *et al.,* 1995) and bacterial system (Wong and McClelland, 1994).

RNA fingerprinting by arbitrarily-primed PCR (RAP-PCR) allows a semi-quantitative comparison of the abundance of several hundred randomly sampled RNAs (Welsh *et al.,* 1992). Three steps are generally required. The first step involves the first strand cDNA synthesis by reverse transcription initiated from arbitrarily-chosen primer at RNA sites best-matched with the primer. Second-strand cDNA was then synthesized by thermal enzymes starting at sites having adequate match on the first-strand cDNA with the same arbitrarily-chosen primer. Next, the cDNA products are further amplified by PCR and resolved by a sequencing gel. 10-50 bands can be generated per fingerprint. The differences in the pattern of bandings reflect the differences in abundance of individual RNAs. As one gel can display many fingerprints, it allows a simultaneous comparison of several hundred RNAs. The protocol did not change much since the first report except some variations in the use of primers. For example, fully degenerated 6-mers oligo were used as primers during the first strand cDNA synthesis followed by two or three longer, arbitrary but defined primers employed in the second strand cDNA synthesis (Sokolov and Prockop, 1994). Notable attempts were also made to bias sampling of a particular gene family like serine/threonine protein kinase or zinc finger family (reviewed in McClelland *et al.,* 1995). In such adaptations, one primer designed according to certain motifs or conserved sequences was introduced in addition to the arbitrarily-chosen primer.

Differential display (DDRT-PCR) differs slightly from RAP-PCR but still involves three basic steps. Using a set of anchored primers, reverse transcription is done in either 4 or 12 fractions. In Liang and Pardee (1992), 12 different primers T_{11} VN were used (where V can be A, G, C and N can be any of the 4 nucleotides). A cDNA fraction generated by each primer represents 1/12 of the total messages. Since the terminal 3' base of the primers provided most of the specificity, the original 12 cDNA fractions was reduced to 4 using 4 degenerate primers T_{12}MN (M is a degenerate mixture of A, G, C) (Liang *et al.,* 1993). Then, second strand cDNA synthesis and subsequent amplification of each fraction was done by using a set of arbitrary primers and anchored primers. Next, the PCR products were displayed by polyacrylmide gel electrophoresis. Up to 50-100 bands can be displayed per lane. Again, the differences of pattern between samples suggest differences in abundance of individual mRNAs. Also, these patterns provide a picture of the mRNA composition. Variations, mainly in the use of primers, from the original protocols were reported. For instances, the anchored oligo-dT primer has been replaced with a second arbitrarily chose primers in the second strand cDNA synthesis (Haag and Raman, 1994). Also, modified long composite primers (10 bases longer) replaced the typical anchor oligo dT primers in the first strand cDNA synthesis (Zhao *et al.,* 1995).

In short, RAP-PCR and DDRT-PCR represent methods that are fast and simple. They allow the identification of differentially expressed genes and detection of individual mRNA species in unlimited number (as far as a gel can handle) of samples. They can also, in principle, represent the whole complexity of messages. Several adaptations have been used to improve the efficiency of the methods such as the introduction of agarose gel (Hsu *et al.,* 1993; Sokolov and Prockop, 1994), chemiluminescent detection (An *et al.,* 1996), automated DNA sequencer (Bauer *et al.,* 1993; Ito *et al.,* 1994) in the display of fingerprints. On the other hand, the methods suffer from non-reproducibility and under-representation of rare mRNA species in general. To tackle non-reproducibility and false positives generated by the methods, various procedures have been carried out (as detailed in section 3.1.4). With regard to the rare species sampling, some authors have been very successful

(Guimaraes *et al.,* 1995). Also, nested priming of fingerprints may improve such sampling. A small aliquot of the original fingerprint is re-amplified with a primer shifted from the original primer 3' and with one or more extra arbitrary bases at the 3' end. These extra bases allow the selection of a subset of the original products including those that are too rare to be observed originally (McClelland *et al.,* 1995).

After comparing and considering the advantages and disadvantages of each protocol for studying differential gene expression (Table 3.1), we found RAP-PCR suit us best. First, five JCS samples of different time point after midazolam incubation were intended to compare at one time. Second, no specific primer oligo is needed to be synthesized as different sequences of primers ranging from 19-24 mers used previously for gene-specific amplifications are readily available in our laboratory. Third, a fast, technically simple method is desired. However, two problems must be solved in order to make this strategy a success. One of them is to eliminate most, if not all, false positive bands (section 3.1.4). Another one is to develop an efficient procedure to reproduce the desired DNA fragment for further cloning and characterizations (section 3.1.3).

Table 3.1. **A comparison of some commonly used methods for studying differential gene expression.**

	Differential Screening	Subtractive hybridization	RAP-PCR	DDRT-PCR
Comparison of mRNA from different sources or time points	Unidirectional (up- or down-regulation)	Unidirectional (up- or down-regulation)	No limitation in the number of samples to be compared, bi-directional	No limitation in the number of samples to be compared, bi-directional
Isolated sequence for further investigation	As cloned sequence (probably full-length cDNA sequence)	As cloned sequence (probably full-length cDNA sequence)	In the form of PCR fragments initially, heterogeneous	In the form of PCR fragments initially, heterogeneous Fragment sequences may cluster towards the 3' untranslated region
Representation of mRNA in the cell	Depends on the number of clones in the cDNA library, intends to represent total mRNA	Represents only mRNAs different between two samples	95 % confidence to acquire the total messages using 18 gels (= 900 primers, 20 mers) (McClelland and Welsh, 1994) Most mRNA should be represented as one band.	90 % confidence of obtaining total messages using 4 different degenerate anchored $T_{12}MN$ * primers X 75 (7-mers) arbitrary primers or 12 $T_{12}MN$ ** primers X 25 (7-mers) arbitrary primers Most mRNA should be represented as one band.
PCR bands generated	Not applicable	Not applicable	Two 20 mer arbitrarily-chosen primers allows a large specificity in sampling the messages (favors longer mRNA) 10-50 bands per fingerprint	Preferentially sample sequence toward the 3' end More bands generated per reaction. (50-100)
Reproducibility	Not generally tested	Not generally tested	Varies with different conditions of reaction	Varies with different conditions of reaction, 20-40 % non-reproducibility (Bauer *et al.*, 1994)

Sensitivity	>0.1 % of messages	0.1 to less than 0.01% messages (Sargent and Dawid, 1983); as sensitive as 0.001 % of messages (Travis and Sutcliffe, 1988)	No data available	Frequency of 1/15,000-1/60,000 messages (Guimaraes *et al.*, 1995)
Sensitivity after introduction of PCR steps	0.03 % of messages (Brunet *et al.*, 1991)	<0.01% of messages, 0.001% of messages (Meszaros and Morton 1996)	No data available	Frequency of 1/15,000-1/60,000 messages (Guimaraes *et al.*, 1995)
Technical difficulties	Moderately difficult	Technically difficult, need high quality, representative cDNA libraries	Relatively easy	Relatively easy
RNA required as starting material	100-500 µg of total RNA, less if PCR techniques are employed	100-500 µg of total RNA, less if PCR techniques are employed	A few to tens of µg total RNA	A few to tens of µg total RNA or corresponding amount of cytoplasmic RNA (Sompayrac *et al.*, 1995) or poly $(A)^+$ mRNA
Time required	Relatively long (months)	Relatively long (months)	Relatively short (days to weeks)	Relatively short (days to weeks)
Others	high background	Hybridization must drive to completion in order to get good results Rare messages have less favorable hybridization kinetics Procedures required to separate non-hybridized and hybridized species. Several rounds of subtraction increase the percentage of enrichment	Procedures required to remove large proportion of false positives and non-reproducibility (see also section 3.1.4) Not limited to poly$(A)^+$ mRNA expression or eukaryotic genes Semi-quantitative	Selected mRNA populations into different groups by anchored oligo-dT primers Procedures required to remove large proportion of false positives and non-reproducibility (see also section 3.1.4) Semi-quantitative

* 4 degenerate $T_{12}MN$: $T_{12}MG$, $T_{12}MA$, $T_{12}MT$, $T_{12}MC$ and M = G, A or C
** 12 $T_{12}MN$: M = G, A or C ; N = G, A,C or T

3.1.3 Re-amplification of PCR products by touchdown PCR

We found that touchdown PCR is an ideal method of avoiding spurious bands or non-specific bands generated during re-amplification procedures without optimization of different components in the reaction.

Touchdown PCR optimizes the reaction entirely by elevated annealing temperature at start followed by decreasing temperature in successive cycles. The originally described amplification profile (Don *et al.*, 1991) started with an annealing temperature well above the estimated melting temperature of the primer-template complexes. The annealing temperature was lowered 1 °C every second cycles for 10 cycles. Finally, the reaction continued with an annealing temperature allowing for the formation of stable primer-template complexes. (5-10 °C below the melting temperature). Various modulations on this protocol are possible (e.g. stepdown PCR with fewer but steeper incremental declines in annealing temperature).

This method/principle has proved to be effective in increasing both the specificity and yield of the PCR (Hecker and Roux, 1996). The priming is initiated well above the optimum annealing temperature (Hecker and Roux, 1996) which favors the amplification of the specific target molecules. As the cycling program progresses, the lowered annealing temperature (< Tm) allows the specific product yield to be increased, which would otherwise be unfavorable by following ordinary thermal profiles.

3.1.4 Strategies to avoid false positives

As mentioned in section 3.1.1 and 3.1.2, RNA fingerprinting by arbitrarily-primed PCR or the related method, differential display has been very useful in isolating differentially expressed genes in different systems. One major problem of these methods is the heterogeneity of the PCR fragments isolated from the resolving gels. Different species of DNA of similar size would migrate as a single amplified band on the gel. Thus, the differentially amplified products of interest (recovered from the gel) are always contaminated with species irrelevant. Also, false positives may be generated by genomic contamination, infrequent imperfect annealing of primers to sequences within the mRNA pool (Rohrwild *et al.,* 1995) and operational errors like degradation of mRNA and inconsistency in pipetting. Three strategies were attempted to avoid these false positives :

1. Comparing fingerprints generated from two or more RNA concentrations for each sample in parallel to eliminate intra-sample variation due to slight differences in the quality or concentration of the RNA (Welsh *et al.,* 1992).

2. Repeating the fingerprints with once or twice new RNA preparation to increase the certainty of detecting real difference in gene expression (Sompayrac *et al.,* 1995).

3. Introduction of primers having a universally pairing base (e.g. inosine) to reduce mis-primings (Rohrwild *et al.,* 1995).

RAP-PCR and DDRT-PCR have been applied to the study of myeloid cells and other hematopoietic cells. Burn *et al.* (1994) studied genes activated by retinoic acid in myeloid cells HL-60. An up-regulated gene, monocyte-chemoattractant protein-1 was isolated and confirmed. Dorn *et al.* (1994) investigated genes differentially expressed during megakaryocyte differentiation into platelets. Both up-regulated genes (Cdw44 adhesion molecules and plectin cytoskeletal protein) and down-regulated gene (glutathione sulfydryl peroxidase) were isolated. Having considered the success of these reports, we employed the strategy of RAP-PCR in studying gene expressions during WEHI-3B JCS cell differentiation triggered by midazolam. The strategy allowed us to monitor the change in mRNA levels at different time points in JCS cells after the drug was added. Since midazolam, different from other inducers, induced granulocytic differentiation in addition to monocytic differentiation of JCS cells, such strategy is certainly valuable in finding and isolating genes related to the phenomenon.

3.2 Materials

Materials which have been mentioned in Chapter 2 will not be presented here.

3.2.1 Cell line and bacterial culture

1. *Escherichia coli* (DH5α)
2. WEHI-3B JCS myeloid leukemia cell line

3.2.2 Chemicals

1. Acetic acid, glacial	Ajax 1
2. Agar	Ajax 863
3. Ampicillin	Sigma A9518
4. Ammonium acetate	Sigma A1542
5. Calcium chloride, hydrated form	Sigma C3881
6. Chloroform	Ajax 152
7. Ethylenediamine-tetraacetic acid (EDTA)	Sigma ED2SS
8. Ethanol, absolute	Ajax 214
9. Glycerol	Sigma G5516
10. Isoamyl alcohol	Ajax 64
11. Isopropyl-β-D-thiogalactopyranoside (IPTG)	Sigma I6758
12. Magnesium chloride	Sigma M9272
13. Magnesium sulfate	Sigma M2773
14. Manganese chloride, tetrahydrate	Sigma M3634
15. Morpholinopropanesulfonic acid (MOPS)	Sigma M8899
16. Rubidium chloride	Sigma R2252
17. Phenol	Sigma P1037
18. Potassium acetate	Sigma P1147
19. Potassium chloride	Sigma P4504
20. Propanol-2-ol / isopropanol	Ajax 425
21. Tryptone	Oxoid L42
22. 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal)	Amresco 7240-90-6

23. Yeast extract	Oxoid L21

3.2.3 Enzymes and nucleic acids

1. ATP	Pharmacia 27-1006-01
2. [α^{32}P]-dCTP	Amersham AA0005
3. pBluescriptSK(-)	Strategene 212206
4. RNase I (10U/μl)	Promega M4261
5. Sma I, 10X buffer J	Promega R612/1,2
6. T4 DNA ligase, 10X One-Phor-All buffer	Pharmacia 27-0870
7. T4 DNA polymerase, 5X buffer	Pharmacia 27-0718

3.2.4 Kits (Appendix A3)

1. Megaprime™ DNA labeling system	Amersham RPN 1606
2. Nick column	Pharmacia Biotech 52-2076-00
3. QIAGEN plasmid midi kit	Qiagen 12143

3.2.5 Solutions

1. LB+Amp^{100} broth	10 g tryptone, 5 g yeast extract and 10 g NaCl per 1L of water, autoclaved at 121 °C under 1 kgcm^{-2} pressure for 20 minutes. A final concentration of 100 μg/ml ampicillin was added to the medium cooled to hand-tolerable temperature
2. LB+Amp^{100} plate	LB+Amp^{100} broth + 10 g/L agar
3. Phenol, equilibrated	5 ml phenol aliquot from -20 °C storage was equilibrated with equal volume of 1 M Tris-HCl, pH 8.0 at room temperature.
4. Phenol-chloroform-iso-amyl alcohol (PCI)	Freshly equilibrated phenol, pH 8.0, chloroform and iso-amyl alcohol mixed in a proportion of 25 : 24 : 1

5. SOB broth	2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl were first combined and autoclaved for 30 minutes under 1 $kgcm^{-2}$ pressure, 10 mM $MgCl_2$ and 10 mM $MgSO_4$ were then added from a 2 M stock of Mg^{2+} filter-sterilized through a 0.22 μm membrane. The medium was used within 3 weeks
6. SOB agar plate	SOB broth + 10 g/L agar
7. RF1 solution	100 mM RbCl, 50 mM $MnCl_2 \cdot 4H_2O$, 30 mM potassium acetate, 10 mM $CaCl_2$•2H2O, 15% (w/v) glycerol, pH 5.8 adjusted by acetic acid and filter-sterilized by 0.22 μm membrane
8. RF2 solution	10 mM MOPS, 10 mM RbCl, 75 mM $CaCl_2.2H_2O$, 15% (w/v) glycerol, pH 6.8 adjusted by NaOH and filtered through a 0.22 μm membrane.
9. $T_{10}E_{0.1}$ buffer, pH 7.5	10 mM Tris-HCl, pH 7.5; 0.1 mM EDTA, pH 7.5
10. $T_{10}E_{0.1}$ buffer, pH 8.0	10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA, pH 8.0

3.3 Methods

3.3.1 Isolation of total RNA

Total RNA was isolated from midazolam-induced JCS cells using the same method as described in section 2.3.1. JCS cells were incubated with 10 μg/ml midazolam for 0, 1, 5, 18 and 48 hours before harvest. The cell culture was kindly maintained by Mr. Bats, S.W. Wong and Dr. N.K. Mak in the Department of Biology, Hong Kong Baptist University.

3.3.2 First strand cDNA synthesis

10 μg total RNA from each of the 5 samples of different time points was diluted to 0.1 μg/μl in a total volume of 100 μl. The solution was heated to 65 °C for 10 minutes and quickly chilled on ice. 100 μl of reaction solution from the same 'cocktail' was then added to and well-mixed with each of the RNA solutions, making up the reverse transcription solution (RT solution). The cocktail contained various components in the final concentrations as described: 40 U RNase inhibitor per μg RNA, 0.1 μg oligo dT_{12-18} per μg RNA, 0.5 mM of each dNTP, 1X reaction buffer, 10 mM DTT and 200 U Moloney murine leukemia virus (M-MLV) reverse transcriptase per μg RNA. The final RT solutions were incubated for 1 hour at 37 °C.

3.3.3 RNA fingerprinting by arbitrarily-primed PCR (RAP-PCR)

(i) RAP-PCR

The method was adapted from Welsh *et al.* (1992). cDNA made from 0.1 μg total RNA of each sample was diluted 5 times in a total volume of 10 μl. The diluted templates were boiled for 10 minutes and chilled on ice immediately. After the evaporated droplets at the cap of the tube was collected by centrifugation, a 50 μl PCR

was set up for each sample having a final concentration of 1.5 mM $MgCl_2$, 1X reaction buffer, 0.2 mM of each dNTP, 2 pmol/L of one arbitrarily-chosen primer (ranging form 19 to 24 bases) and 0.25 U Thermoprime$^+$ DNA polymerase in the reaction mixture. 5 low stringency cycles followed by 45 high stringency cycles were performed in a programmable PCR machine (MJ Research). The thermal profile for low stringency cycles was 94 °C denaturation for one minute, (Tm of the chosen primer-20) °C primer annealing for one minute and 72 °C extension for one minute while that for high stringency cycles was 94 °C for one minute, (Tm of the chosen primer -10) °C for one minute and 72 °C for one minute.

(ii) Recovery and re-amplification of PCR fragments

15 µl of the resulting PCR products from the five samples of different time points were loaded in parallels into a 1.5 % TAE Synergel. PCR fragments of different sizes were resolved on the gel. Fragments that were differentially amplified among the samples were cut and recovered from the gel. For each piece of gel, 450 µl of sterilized ultra-pure water (10 times of the gel volume) was added and boiled for 10 minutes. The boiled gel solution with eluted DNA was then stored at -20 °C and used within a week or amplified immediately. 2 µl of the eluted DNA solution was used as template and amplified in a 50 µl PCR reaction with 1.5 mM $MgCl_2$, 1X reaction buffer, 0.2 mM of each dNTPs, 2 pmol/L of the primer originally chosen and 0.25 U Thermoprime$^+$ DNA polymerase. Touchdown amplification profile of 35 cycles was used in this reaction. While the denaturation and extension steps were kept at 94 ° C and 72 °C for 1 minute respectively, the annealing temperature kept for 1 minute was equal to (Tm of the chose primer) °C at start and reduced by 1 °C at each of the following cycles until the fifth cycle. The annealing temperature would be (Tm-5) °C by the fifth cycle and this temperature for primer annealing was kept for further 30 cycle amplification. Two identical reactions were prepared for each fragment isolated and the two final reaction solutions were combined into one tube at the end.

3.3.4 First round cDNA probe screening

(i) Preparation of cDNA probe

5 μg of total RNA from each of the 5 samples was reverse-transcribed as described previously (section 3.3.2), except that each reaction components was scaled down accordingly to obtain the first strand cDNA. The 100 μl of reverse-transcribed reaction solution from each sample was added with equal volume of PCI and vortexed thoroughly. The aqueous and the organic phases were separated by brief centrifugation (Eppendorf). The upper aqueous phase of about 100 μl was pipetted out and transferred to a new tube. 55 μl of 7.5 M ammonium acetate and 400 μl of absolute ethanol were added to precipitate the nucleic acids. The solution was well-mixed and spun at 14 000 rpm on a bench-top microcentrifuge at room temperature for 30 minutes. The pellets were washed with 70% ethanol and resuspended in 50 μl of $T_{10}E_{0.1}$ buffer, pH 8.0. The solution obtained from the 5 different time points were stored at -20 °C until use.

(ii) ^{32}P-labeling

To label cDNAs in the RT solution with ^{32}P, random-primed DNA labeling was used. By using the Megaprime labeling system (Amersham) following the new megaprime protocol, two 50 μl reactions were set up for each type of RT solution. Each reaction contained 20 μl of the RT solution prepared in (i) (derived from 2 μg total RNA), 5 μl of random nanomers, 10 μl of labeling buffer, 2 μl of DNA polymerase, Klenow fragment, 6 μl of [α^{32}–P]-dCTP of radioactivity 10 μCi/μl (=20 pmol, specificity activity 3000 Ci/mmol) and 7 μl of H_2O making up the reaction volume to 50 μl (see also Appendix A3). Master mix I containing primers and H_2O was first dispensed to each of the 20 μl of RT solution. The tubes were then boiled for 5 minutes, spun down and cooled down to room temperature for primer annealing. Master mix II with labeling buffer, enzyme and radioactive label was added to each reaction subsequently and mixed thoroughly by pipetting up and down. The final

labeling reaction solution was spun down and incubated for 10 minutes at 37 °C. The two identical reactions for the same probes were pooled together and 4 µl of 0.5 M EDTA, pH=8.0 was added to stop the polymerization.

(iii) Probe purification by nick column

The labeled DNA from the previous section can be purified by nick column (Pharmacia). 3 ml of equilibration buffer, $T_{10}E_{0.1}$ pH 7.5 was applied to the column until it entered the gel bed completely. The total 100 µl of each labeling reaction mix was applied at the centre of the filter on the top of the column and let to enter the gel bed. The first elution run-out fraction by adding 400 µl of $T_{10}E_{0.1}$ pH 7.5 buffer was discarded but the second eluted (400 µl of $T_{10}E_{0.1}$ buffer) fraction containing most of the labeled DNA was collected.

(iv) Pre-hybridization and hybridization

PCR products obtained in section 3.3.3 was further amplified by PCR. Each of the products was dot-blotted in equal amount onto 5 sets of nylon membranes. A 'control dot' having equal amount of a GAPDH fragments amplified by specific primers (Table 2.1) was also applied to the membranes where the hybridization signals were compared. The membrane was fitted in the dot-blot apparatus according to the instructor's manual (see also Figure 2.2). 200 µl of H_2O was added to each well to pre-wet the membrane and drained using vacuum force. 15 µl of each PCR amplified product was first denatured in 0.2 M NaOH in a total volume of 200 µl solution for 15 minutes at room temperature and applied to each well under vacuum. 400 µl of 20X SSC was then added to each well to wash off any sticky droplets on the wall of the well and to neutralize the membrane. The membrane was removed from the dot-blot apparatus, air-dried and baked for 30 minutes at 120 °C. The membranes were stored in cool, dry place at room temperature until use.

Before hybridization, the membranes were completely wet with hybridization buffer. The membranes destined to the same time point of cDNA probe were curled

up together with meshes in between. The curled membranes were put into an appropriate glass tube (Hybriad) pressed against the wall by a glass pipette in a rotatory manner opposite to the curl. 20 ml of hybridization buffer was added to each tube and pre-hybridized for 10 hours at 68 °C in a hybridization oven (Hybriad). The used buffer was then replaced with 10 ml of new buffer. 400 µl of the purified radioactively-labeled probe of each time point, namely 0, 1, 5, 18 and 48 hour, was then added at the centre of the buffer, swirled to mix and rotated to ensure complete coverage of the membrane. The membranes were hybridized for 18 hours at 68 °C with continuous rotation. About 20 ml of 2 X SSC; 0.1 % (w/v) SDS was added to each tube at 68 °C for two stringent washes, each for 15 minutes. Another two washings were done using 0.5 X SSC; 0.1% (w/v) SDS at 68 °C each for 15 minutes. The membranes were then removed from the tubes, sealed with Saran wrap and exposed to the same piece of X-ray film (Kodak 165-1512) at room temperature in an X-ray film cassette (X-Omatic cassette, Kodak) for 12 hours at room temperature. The film was developed and fixed to obtain an autoradiograph.

(v) Quantitation by densitometry

Dots on the autoradiograph were scanned by a densitometer (Bio-Rad) and quantitized in integrated volume = [OD X area (mm^2)] using Volume Analysis in the Molecular Analyst^{TM} software (Bio-Rad). Background subtraction was done as described in section 2.3.8. After background subtraction, the value of the 'control dot' on membranes of different time points were normalized. The value of other dots on each membrane was then adjusted in direct proportion to this normalized value of the control. Dots showing a 2-fold difference (or more than a 2-fold difference) in the normalized values among samples of different time points were picked for further study.

3.3.5 Subcloning of differentially amplified fragments

(i) Preparation of vectors

25 ml of DH5α-pBluescript SK(-) overnight culture using LB+Amp^{100} broth was prepared at 37 °C. Plasmid DNA was isolated using QIAGEN plasmid midi kit (Appendix A3). Briefly, the bacterial cells were pelleted at 7000 rpm (6000 g) in a JA-20 rotor (Bechman) for 15 minutes at 4 °C. The supernatant was decanted and the tube was inverted to drain on absorbent paper. The pellets were resuspended in 4 ml of P1 solution added with 20U RNase I. 4 ml of P2 solution was then added and mixed by gentle inversion (4 times) to lyse the cells. The mixture was incubated for 5 minutes at room temperature and 4 ml of P3 solution was added to neutralize the cell lysate by immediate gentle inversion (4 times). The neutralized lysate was then transferred to the barrel of a 10 ml syringe which is connected to a filter unit with a piece of large pore filter (Millipore AP1002500) outlet to the Qiagen-column (Figure 3.1). The column was equilibrated with 4 ml of QBT solution and allowed to empty by gravity flow just before use. The lysate was settled in the set-up at room temperature for 5 minutes before the stopper was removed. The plunger of the syringe was then pushed through to filter out the precipitates. The lysate was let to enter the resin of the column under gravitational force. The column was then washed twice, each with 10 ml of QC solution. The DNA was eluted out with 5 ml of QF solution. The eluted DNA was precipitated with 3.5 ml of isopropanol at room temperature and centrifuged immediately in a JA-20 rotor (Bechman) at 12 000 rpm (15 000 g) for 30 minutes at 4 °C. The resulting supernatant was removed and the pellets were washed with 2 ml of 70 % ethanol. The washed pellets were further centrifuged at 12 000 rpm (15 000 g) in a JA-20 rotor for 10 minutes at 4 °C. The supernatant was discarded and the pellets were vacuum-dried (Savant) and resuspended in 400 μl of H_2O. The solution was then analyzed and quantitized by spectrophotometry at 260 nm and 280 nm UV light. 1 O.D. unit at 260 nm was taken as 50 μg/ml.

20 μg of plasmid DNA was digested by Sma I (blunt-end digestion) for 1 hour at 25 °C in a total volume of 200 μl. Plasmid DNA concentration was kept at 0.1

μg/μl and Sma I enzyme concentration was 1.5 U/μg DNA. After the digestion, 200 μl of PCI was used for purification. The mixture was vortexed thoroughly and centrifuged to separate the two phases. The upper phase was pipetted out to a new tube. 110 μl of 7.5 M ammonium acetate and 775 μl of 100 % ethanol were added for precipitation. The solution was centrifuged at 14 000 rpm on a bench-top microcentrifuge (Eppendorf) for 30 minutes. The supernatant was discarded and the pellets were washed with 1 ml of 70 % ethanol and vacuum-dried (Savant). The dried pellet was resuspended in 350 μl of ddH_2O making up to a final concentration of about 0.05 μg/μl of DNA.

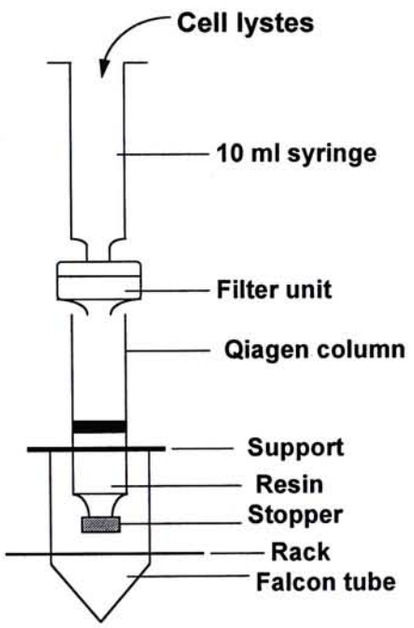


Figure 3.1. **Set-up for plasmid purification.**

(ii) Preparation of inserts

PCR fragments showing a change of about two-fold difference or more in hybridization signal among the time points were further amplified by PCR as described in section 3.3.3 using 2 μl of PCR products as template. Two PCR (50 μl) for each fragment were performed and the resulting products were combined afterwards. One PCI extraction was done and the PCR products were precipitated by 60 μl of 7.5 M ammonium acetate and 375 μl of 100 % ethanol. The solution was

centrifuged at 14 000 rpm for 30 minutes and the pellets were washed once with 70 % ethanol. The pellets were vacuum-dried (Savant) and resuspended in 55 µl of ddH_2O. 11 µl of this purified PCR products was first mixed with 4 µl of 5 X buffer and 10 U of T4 DNA polymerase and waited exactly for 30 seconds. Then, 4 µl of 10 mM each dNTPs was added making the final concentration to 2 mM each dNTP and final volume to 20 µl. The mix was incubated for 30 minutes at 37 °C, spun down and chilled on ice. 20 µl of PCI was added, mixed and the upper aqueous layer was pipetted out to a new tube. 10 µl and 75 µl of 7.5 M ammonium acetate and absolute ethanol were added respectively and the tube was spun at 14 000 rpm for 30 minutes (Eppendorf). The supernatant was removed and the pellets were washed with 0.5 ml of 70% ethanol and dried in vacuum. The dried pellets were resuspended in 10 µl of H_2O.

(iii) Blunt-end ligation

4 µl (about 0.2 µg) of pBluescript/Sma I prepared in section 3.3.5(i) was mixed with 10 µl of blunt-ended PCR fragments obtained in section 3.3.5(ii), 2 µl of 10 X One-Phor-All buffer, 2 µl of 10 mM ATP and 16 Weiss Unit of T4 DNA ligase making up the final volume to 20 µl. The reaction mix was incubated in a PCR machine for 16 hours at 16 °C (MJ Research). A PCI extraction was done and the nucleic acids were precipitated with 10 µl of ammonium acetate and 75 µl of absolute ethanol followed by centrifugation at 14 000 rpm for 30 minutes (Eppendorf). The final pellets were washed with 0.5 ml of 70% ethanol, vacuum-dried (Savant) and resuspended in 15 µl of H_2O.

(iv) Preparation of competent cells

Several DH5α colonies 2-3 mm in diameter were picked from a SOB agar plate, suspended in 1 ml of SOB broth and grown for 18 hours at 37 °C. The culture was then diluted in 99 ml of SOB medium in a 2000 ml flask. The diluted culture was then incubated with moderate agitation at 37 °C until the cell density equals to 4-7 X 10^7 /ml. For DH series, this cell density corresponds to an absorbance of 0.45-0.55 at

550 nm. The absorbance of the growing culture was checked at a 30 minute interval and the cell density required reached after about 2 hours. The culture was collected and incubated on ice for 15 minutes. The cells were then harvested at 3000 rpm in a JA-20 rotor (Bechman) for 15 minutes at 4 °C. The supernatant was discarded and the remaining droplets were absorbed on tissue paper upon inversion. The cells were then resuspended with 30 ml of RF1 solution and mixed moderately by vortex. The cell suspensions were incubated on ice for 15 minutes and centrifuged at 3000 rpm in a JA-20 rotor (Beckman) for further 5 minutes at 4 °C. The cell pellets were dispersed in 8 ml of RF2 solution and incubated on ice for 15 minutes. The resulting cell suspension was then pipetted in 110 µl aliquot by pre-chilled pipette tips into pre-chilled screw cap tubes. The tubes were then flash-freezed by liquid nitrogen. These cells were made competent and stored at -70 °C until use.

(v) Transformation

An aliquot of competent cells (110 µl) removed from -70 °C was thawed at room temperature until the cell suspension become just liquid. The 15 µl of ligated plasmids prepared as described in section 3.3.5 (iii) was added to the cell suspension and swirled to mix evenly. The tube was kept on ice for 1 hour. The cells were then heat-shocked by placing the tube in a 42 °C water bath for 90 second and chilled on ice immediately afterwards. 800 µl of pre-warmed LB broth was then added and incubated at 37 °C for another hour. The cells were then plated out on LB+Amp^{100} plates with 8 µl of 0.5 M IPTG and 4 µl of 250 mg/ml X-gal in a volume of 50 µl, 100 µl or 200 µl and incubated at 37 °C overnight. The white colonies were spotted and further studied.

(vi) Selection, screening and confirmation by PCR

10 white colonies were picked randomly on LB+Amp^{100} plates with IPTG and X-gal as described in section 3.3.5(v). The picked colonies were spotted on a master LB+Amp^{100} plate and at the same time each colony was resuspended in 100 µl of H_2O. The colonies were named according to the primer number, fragment number and

colony number (e.g. 44/3.1). The master plate incubated at 37 °C overnight and the resuspended colonies were boiled for 10 minutes, chilled on ice and spun for 1 minute. 10 µl of each bacterial lysate was used as template for PCR amplified for 50 cycles following the thermal profile : 94 °C for 1 minute, 56 °C for 1 minute and 72 °C for 1 minute. The products were analyzed on a 1.5 % TAE Synergel and those which showed different sizes on the gel were selected for a second screening. The master plate were kept at 4 °C as stock and spotted onto a new plate twice a month.

3.3.6 Second round cDNA probe screening

The screening followed basically the procedure described in section 3.3.4 except that the PCR fragments to be screened were generated using the plasmids with the subcloned fragments as templates. The resulting products were then further amplified and dotted onto the membranes. As in the first screening, fragments with normalized value having 2-fold difference or more among samples of different time points were selected for further characterization.

3.4 Results

3.4.1 Spectrophotometric analysis of total RNA

Total RNAs from 6 different samples (Table 3.2) obtained by the guanidinium thiocyanate-cesium chloride isopycnic gradient method (Chirgwin *et al.*, 1979) were analyzed by measuring the absorbance of UV light at 260 nm and 280 nm. The ratio of the 2 values defined an approximate purity of the samples. In these preparations, the ratio was roughly the same.

Table 3.2. **Spectrophotometric analysis of the RNAs isolated from 6 different samples after midazolam treatment.**

Sample	A_{260}/A_{280}
Control JCS cells (Batch 1)	1.53
Control JCS cells (Batch 2)	1.56
JCS cells incubated with midazolam (10 μg/μl) for 1 hour	1.54
JCS cells incubated with midazolam (10 μg/μl) for 5 hours	1.41
JCS cells incubated with midazolam (10 μg/μl) for 18 hours	1.53
JCS cells incubated with midazolam (10 μg/μl) for 48 hours	1.54

Total RNAs were prepared from untreated JCS cells and JCS cells incubated with 10 μg/ml midazolam for 1, 5, 18 and 48 hours. A_{260} and A_{280} are the absorbance of UV light at 260 and 280 nm respectively. The ratio of the two values were calculated as shown.

3.4.2 Normalization of samples

The 6 samples of different treatments were normalized by GAPDH gene expression detected by RT-PCR amplified for 20 and 25 cycles followed by electrophoresis on a 1.5 % TAE Synergel. The expression of GAPDH was essentially the same among the samples under the detection (Figure 3.2).

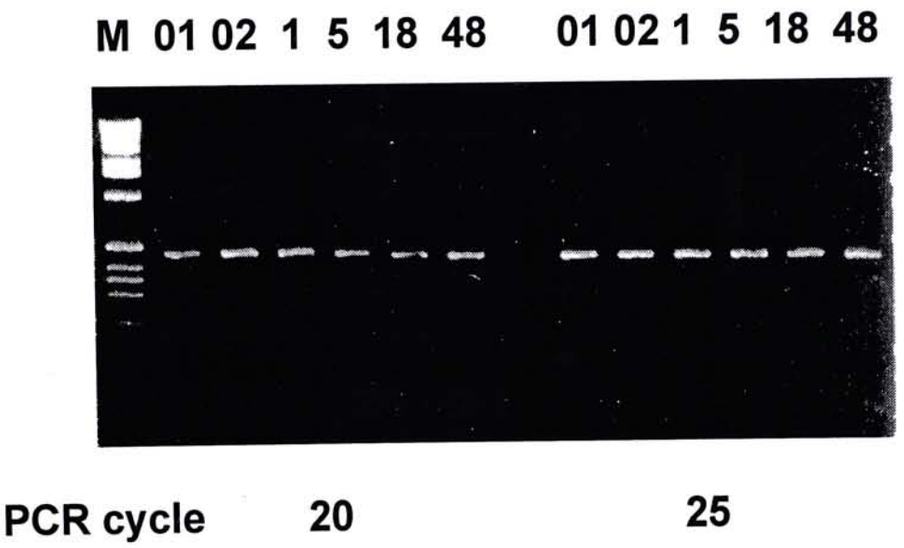


Figure 3.2. **Normalization of samples by GAPDH gene expression.** 20 and 25 cycles of RT-PCR using GAPDH gene specific primers were performed on RNA samples isolated from untreated JCS cells (Batch 1 (01) and Batch 2 (02)) or JCS cells incubated with midazolam (10 μg/ml) for 1 (1), 5 (5), 18 (18) and 48(48) hours. 10 μl of each of the PCR products were electrophoresed on a 1.5 % TAE Synergel. The expected product size was 452 bp. M = 1Kb ladder.

3.4.3 RNA fingerprinting by arbitrarily-primed PCR

Six populations of polymorphic fragments generated from total RNA samples were amplified with a modified RAP-PCR protocol as described in section (3.3.3(i)). The arbitrarily primers chosen were 19-24 bases long. The RNA fingerprints were size-fractionated on a 1.5 % TAE Synergel and run side-by-side for comparison. Different kinds of polymorphism were observed as exemplified in Figure 3.3. Some fragments were only amplified in samples of early hours and peaked at the time point 5 (Figure 3.3, arrow 1 and 3). Other polymorphic bands were abundant at 0 and 1 hour samples and in lower abundance at later time points (Figure 3.3, arrows 2 and 3). There were also bands that appeared exclusively in samples of 0 and 1 hour (Figure 3.3, arrow 5). A total of 44 primers have been used to generate 142 such differentially amplified fragments. 0-9 polymorphic bands were produced per arbitrarily-chosen primer and an average of 3 bands could be generated per primer. The number of resolved bands per fingerprint ranged from 1 to 12 bands and most of them gave 6-9 bands. The size of the resolved bands distributed between the 201 and 2036 bp marker band of the 1Kb ladder. It was also noted that different batches of untreated JCS cell sample resulted in different banding pattern at the same measured concentration. Unless otherwise stated, the bands determined to be polymorphic were based entirely on the untreated JCS cells-batch 2 and samples of the other time points.

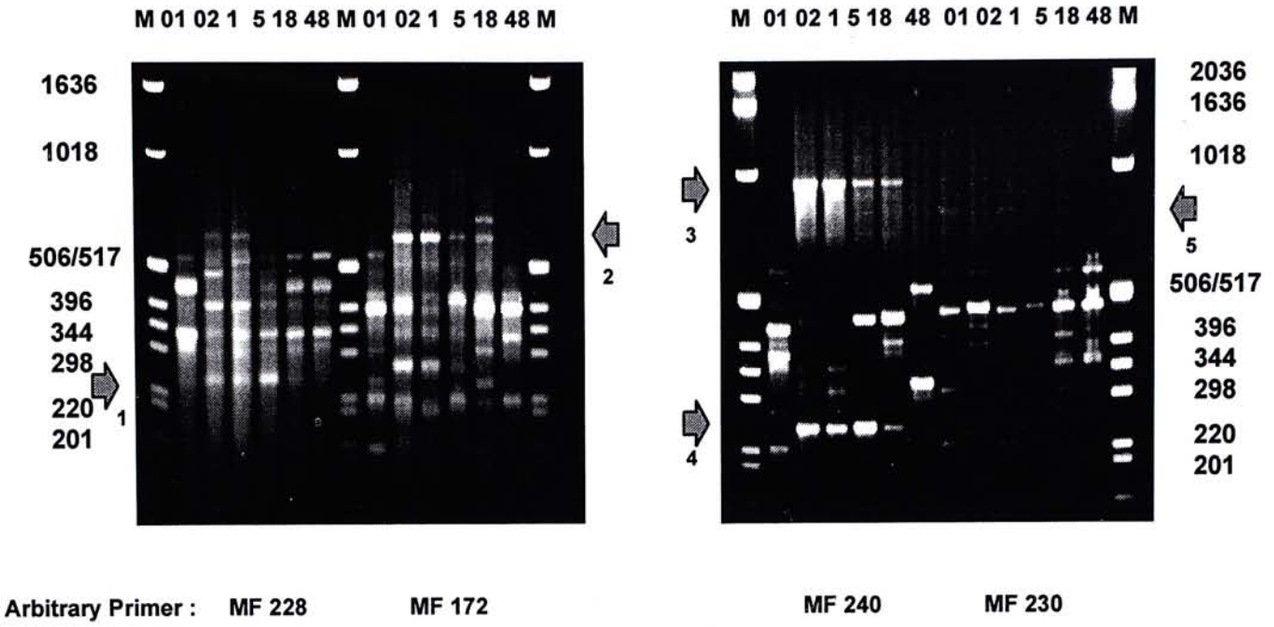


Figure 3.3. **RNA-fingerprinting by arbitrarily-primed PCR.** Six populations of differentially amplified fragments were generated by RAP-PCR using total RNAs from control JCS cells (batch 1 (01) and batch (02)) and JCS cells incubated with midazolam (10 μg/ml) for 1, 5, 18 and 48 hours. For each set of reaction, 5 low stringency cycles and 45 high stringency cycles were performed with the arbitrarily-chosen primers indicated. The resulting products were loaded into a 1.5 % Synergel. The numbers on the side of the photo are the size of the marker bands. Some of the polymorphisms among the samples were indicated by arrows and recovered as described in section 2.3.3(ii). M=1Kb ladder.

MF 228 5′ AGA AGT GAA GGC TGG CAT GGC 3′

MF 172 5′ ATG GAA GGG ATG ACT ATG TTG GA 3′

MF 240 5′ TCA TCA ACC ACC TTC GAA AAT AGC 3’

MF 230 5′ GAA TGT CCA CAA ACT GAT ATG CTT 3′

3.4.4 Re-amplification of PCR product

The differentially amplified bands were directly recovered from the gels under UV illumination. The small pieces of gel were boiled immediately for 5 minutes and 2 μl of the eluted and diluted DNA solution was re-amplified by touchdown PCR as detailed in section 3.3.3(ii). The re-amplified products were shown in Figure 3.4. While most of the products remained homogenous in size (Lane 1-6, 8-12), some other products gave more than one dominant band after re-amplification (Lane 7, 13-16).

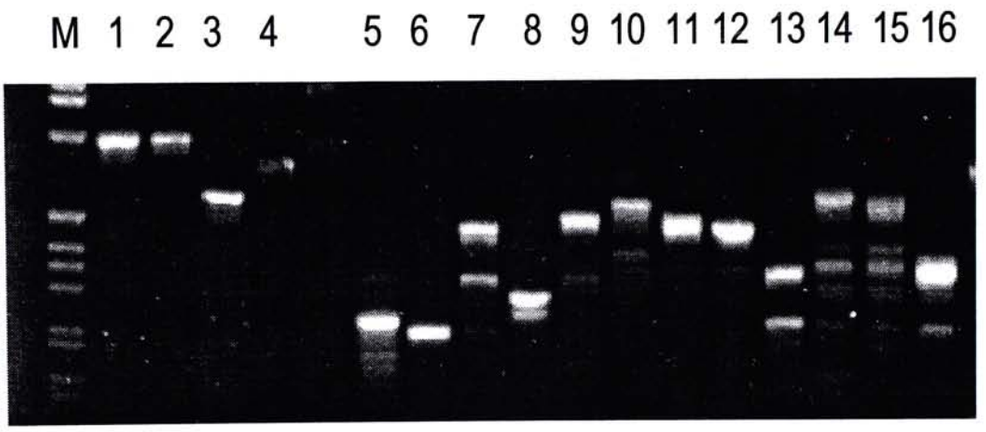


Figure 3.4. **Re-amplification of the differentially amplified fragments isolated.** The fragments that were differentially amplified on the RNA fingerprints were cut and eluted by boiling. 2 μl of the eluted and diluted (10 times) DNA solution was re-amplified by 35 cycles of touchdown PCR using the original arbitrarily-chosen primers (section 3.3.3(ii)) and the products were visualized on a 1.5 % TAE Synergel. Lanes 1-6, 8-12 showed some homogeneous products after amplification while the rest (7, 13-16) illustrated products which were more heterogeneous. Most of these isolated fragments have size fell in the range of 201-1018 bp. M=1Kb ladder.

3.4.5 First round cDNA probe screening

The re-amplified PCR products of each isolated band, despite their heterogeneity was dot-blotted in equal amounts onto 5 sets of membranes. Each set of the membranes was then hybridized with one type of the cDNA probes derived from the total RNAs of JCS cells treated with 10 μg/ml midazolam for 0, 1, 5, 18 or 48 hours (see section 3.3.4) (Figure 3.5). The hybridization signal of each dot was scanned and converted into numerical value by densitometer and the affiliated software (see section 3.3.4(iv)). The converted values were further normalized according to the control dot GAPDH. The slopes between values of the GAPDH dots were established and all other values were adjusted according to the slopes. The two membranes of each set were normalized separately with respect to individual control dot on the membranes. Referring to the normalized value, fragments that have corresponding PCR products showing a change in hybridization signals of 2 folds or more than 2 folds across the time points were subjected to further analysis. Table 3.3 showed all the selected PCR fragments. The expression profile as presumed from the normalized values of the selected fragments can be summarized into 3 types (Figure 3.6). Of the 10 fragments examined, one fragment was found to have slight transient up-regulation at 1 hour followed by down-regulation (A). Six of them were found down-regulated at the time point 18 hours (B). Finally, three of them were steadily up-regulated throughout the treatment (C).

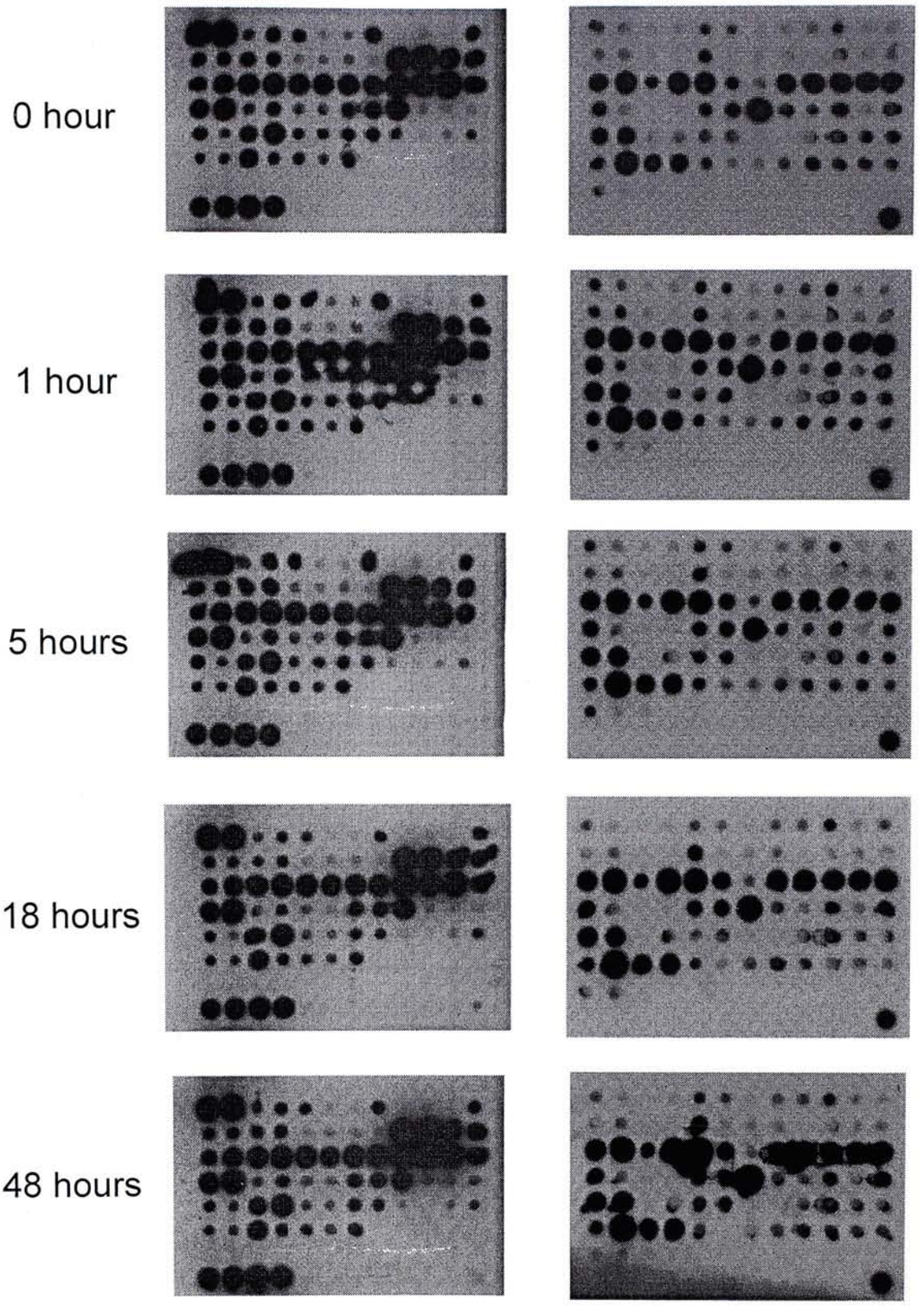


Figure 3.5. **First round cDNA probe screening.** 142 differentially amplified bands on the RAP-PCR gels were recovered and re-amplified. The re-amplified products and control GAPDH fragment (for normalization) were dotted in equal amounts onto 5 sets of membranes. ^{32}P- labeled cDNA probes derived from total RNAs of midazolam-induced JCS cells of different time points as indicated were each hybridized with one of the membrane sets for 18 hours at 68 °C. The hybridization signals were visualized by autoradiography (12 hour exposure at room temperature) and analyzed by densitometric scanning (section 3.3.4 (iv), Table 3.3).

Table 3.3. **Hybridization signals in numerical values resulted from the first round cDNA probe screening**

Fragment	Volume = [OD X area (mm^2)]										Expected expression pattern *
	Time point (hours)										
	Raw data					Normalized data					
	0	1	5	18	48	0	1	5	18	48	
GAPDH (1)	25.28#	27.50#	26.54	25.77	29.54	25.28	25.28	25.28	25.28	25.28	-
44/3	8.57	13.49#	7.32	1.33	1.84	8.57	12.40#	7.00	1.30	1.57	A
147/1	10.19	12.00	11.45	1.96	5.83	10.19	11.03	10.90	1.93	5.00	B
240/3	13.19	14.61	13.80	3.54	8.20	13.19	13.43	13.14	3.47	7.02	B
172/2	9.16	17.22	12.52	4.72	7.84	9.16	15.83	11.93	4.63	5.71	A
172/3	10.40	15.49	12.85	4.84	9.37	10.40	14.24	12.24	4.75	8.02	B
172/4	11.30	14.68	13.77	2.97	7.36	11.30	13.50	13.11	2.91	6.30	B
172/5	10.00	14.53	13.76	3.31	7.07	10.00	13.36	13.11	3.25	6.05	B
GAPDH (2)	30.51	29.04	24.08	19.55	29.13	19.55	19.55	19.55	19.55	19.55	-
230/5	12.24	12.58	13.86	13.95	25.07	7.84	8.47	11.26	13.95	16.83	C
228/2	13.64	18.19	13.99	13.89	21.39	8.74	12.25	11.36	13.89	14.35	C
228/5	13.22	20.95	17.51	18.40	27.13	8.47	14.11	14.22	18.40	18.20	C

Hybridization signal of each dot from the first round screening was scanned and converted into numerical values which was then normalized according to the value of the control dot GAPDH (see also text). GAPDH (1) and GAPDH (2) corresponds to control dots on 2 different membranes. Fragments selected for further analysis were presented here. cDNA probes were made from total RNA of the JCS cells having been incubated with midazolam (10 μg/ml) for 0, 1, 5, 18 or 48 hours.

* Expression pattern A : Slight transient up-regulation at 1 hour followed by down-regulation. B : Constitutive expression at early hours followed by down-regulation at the time point 18 hours. C : Steady up-regulation during the midazolam-induced differentiation. see also Figure 3.6.

Illustration of value adjustment : The normalized data 12.40 was calculated by multiplying the raw data 13.49 to the slope of the raw GAPDH dot value for 0 hour against that for 1 hour. (25.28/27.50)

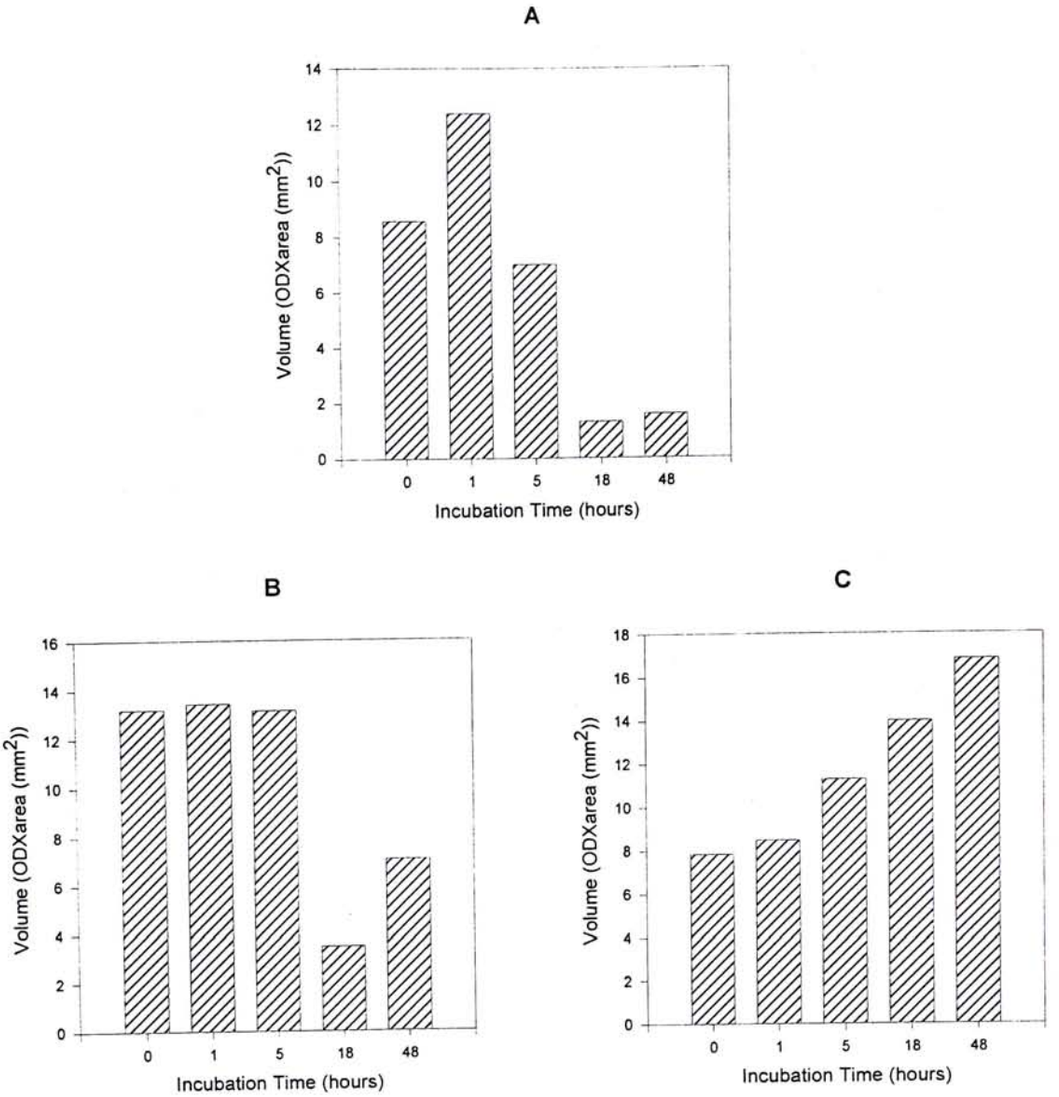


Figure 3.6. **Expression patterns of the selected gene fragments deduced from the first round cDNA probe screening in midazolam-treated JCS cells.** Three major expression patterns of the isolated differentially amplified fragments were observed. A. Slight up-regulation at a 1 hour followed by dramatic down-regulation starting at the 5th hour. B. Constitutive expression at early hours followed by down-regulation at the time point 18. C. Steady up-regulation during the incubation.

3.4.6 Subcloning of the differentially amplified fragments

The differentially amplified gene fragments (Table 3.3) were subcloned into the pBluescript SK- vector using the corresponding re-amplified products (see section 3.3.5). Ten randomly picked white colonies labeled as clone .1 to .10 from LB+Amp^{100} plates were lysed in sterilized H_2O. The lysates were used as templates for standard PCR using the original arbitrarily-chosen primers (see section 3.3.5 (vi)). All the colonies were confirmed to have inserts and examples were shown in Figure 3.7. However, the ten 'clones' resulted from the same preparation of subcloning and transformation may have inserts of the same size (Figure 3.7, 44/3), slightly different sizes (Figure 3.7, 228/5 and 147/1) or very different sizes (Figure 3.7, 230/5) on gel analysis. Clones that appeared to have different sizes were considered unique and treated individually. Each of these fragments were re-amplified by touchdown PCR and undergone a second round of cDNA probe screening.

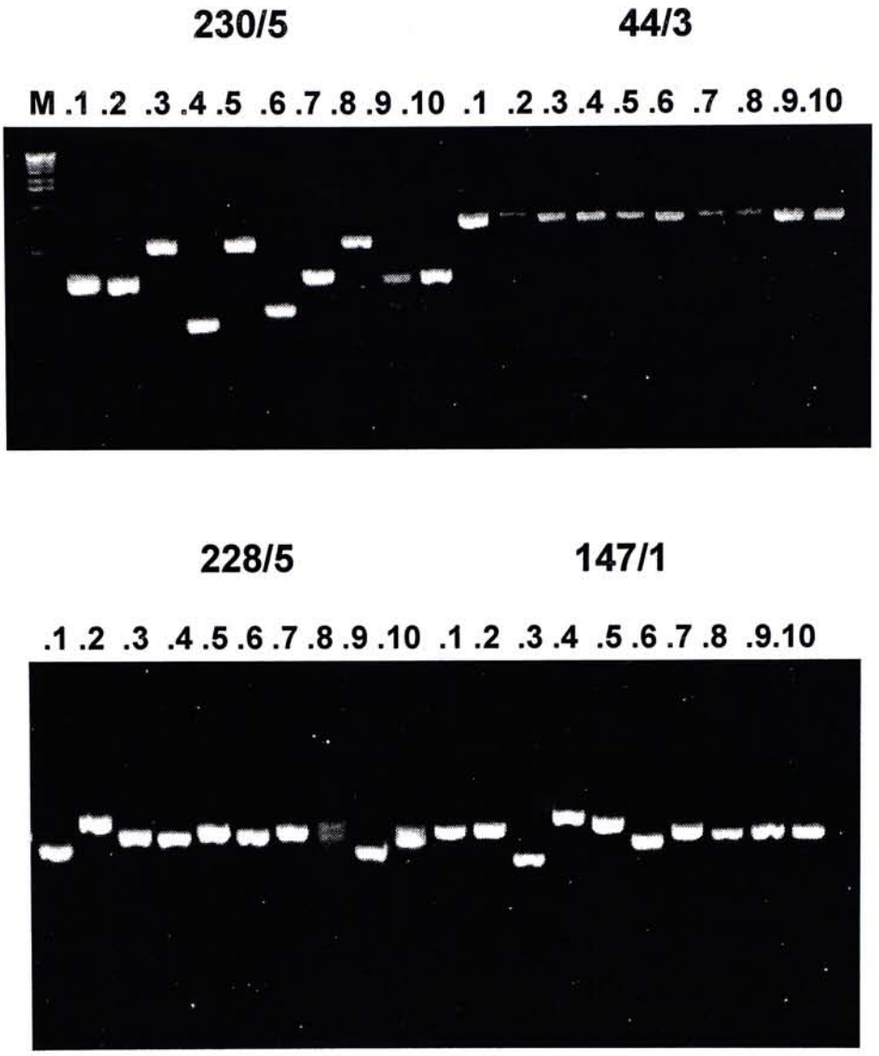


Figure 3.7. **Subcloning of the differentially amplified gene fragments and confirmation by 50 cycles of PCR.** 10 randomly chosen white colonies on LB+Amp ^{100} + IPTG + X-gal plate (section 3.3.5(vi)) were picked and lysed in double distilled water as described in methods. 10 µl of the lysate was used as template for 50 cycles of PCR and the products were analyzed on a 1.5 % TAE Synergel. Different colonies ('clones') may have recombinant plasmids harboring inserts of the same size (44/3 series), slightly different sizes (228/5 and 147/1 series) or entirely different sizes (230/5 series). M=1Kb ladder.

3.4.7 Second round cDNA probe screening

The dot-blotted PCR products corresponding to the subcloned fragments were hybridized by ^{32}P-labeled cDNA probes as in the first round screening (Figure 3.8). In contrast to the previous one, each dot of signal was resulted from a single homogenous DNA fragment in this screening. As before, the signals were presented in numerical terms, normalized with reference to the 'control dot' GAPDH (Table 3.4). Subcloned fragments with PCR products showing significant changes (2-fold) in hybridization signals across the time points were further characterized. The expression patterns of these subcloned fragments were also analyzed based on the normalized value of the hybridization signal. Four patterns were observed (Figure 3.9 and Table 3.4) A. Constitutive expression at early hours of midazolam treatment but the level down-regulated starting at the 5th hour (8 fragments). B. Steady down-regulation (3 fragments). C. Transient up-regulation at 1 hour (2 fragments) and D. Steady up-regulation (4 fragments). The expression patterns for uninterpretable dots were determined by visual examination.

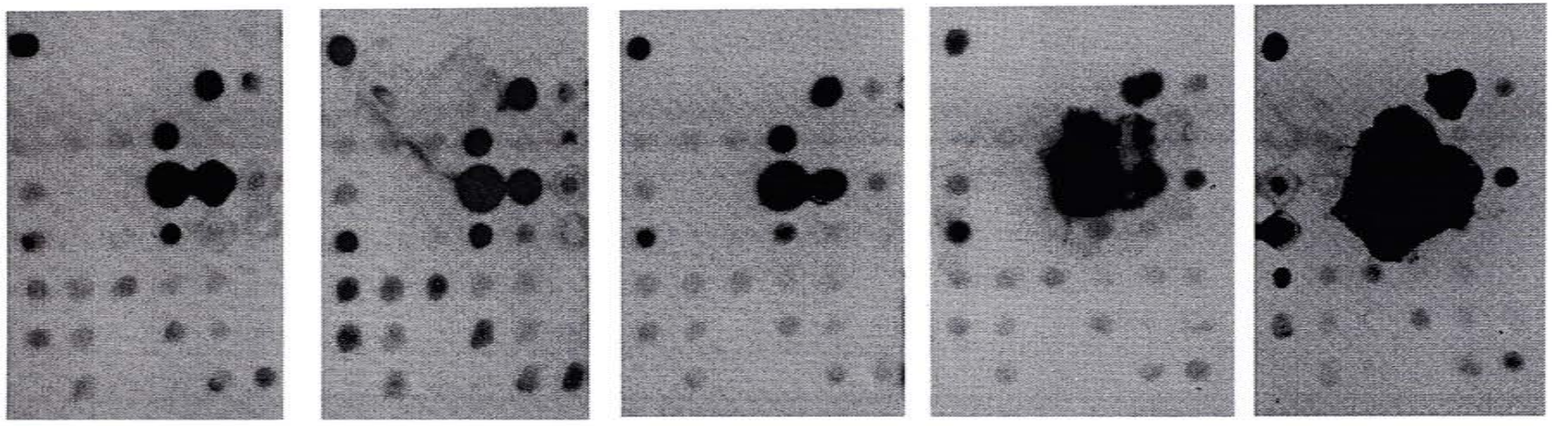


Figure 3.8. **Second round cDNA probe screening.** Colonies having recombinant plasmid of various sizes of inserts as shown in Figure 3.7 were considered to be different 'clones'. These inserts of different sizes were re-amplified by touchdown PCR and dotted separately in equal amount onto 5 membranes. ^{32}P-labeled cDNA probes generated from total RNAs of midazolam-induced JCS cells of different incubation time as indicated were used to hybridize with these membranes for 18 hours at 68 °C. An autoradiograph was developed on X-ray film exposed to the membranes for 12 hours at room temperature. The hybridization signals of each dot were analyzed by densitometric scanning (section 3.3.4 (iv), Table 3.4)

Table 3.4. **Hybridization signals in numerical values resulted from the second cDNA probe screening.**

Subcloned fragment	Volume = [OD X area (mm^2)]										Expected expression pattern *
	Time point (hours)										
	Raw data					Normalized data					
	0	1	5	18	48	0	1	5	18	48	
GAPDH	24.11	25.34	28.40	27.27	31.85	24.11	24.11	24.11	24.11	24.11	-
44/3.1	14.35	12.97	9.70	7.44	10.38	14.35	12.35	8.24	6.58	7.86	A
147/1.4	14.91	18.72	11.99	20.64	33.32	14.91	17.82	10.18	18.26	25.23	D
147/1.5	6.04	3.88	1.88	2.06	3.35	6.04	3.69	1.60	1.83	2.53	B
147/1.6	1.05	1.04	0.62	0.02	2.31	1.05	0.99	0.52	0.02	1.75	B
172/2.8	4.05	8.58	1.86	1.53	5.30	4.05	8.16	1.58	1.35	4.02	C
172/3.1	3.37	5.81	1.15	1.43	2.68	3.37	5.53	0.98	1.26	2.03	A
172/3.2	5.01	6.93	2.09	1.95	4.16	5.01	6.60	1.77	1.73	3.15	A
172/3.8	4.81	8.44	2.33	1.30	4.15	4.81	8.04	1.98	1.15	3.15	C
172/4.1	2.66	2.87	1.18	0.74	1.54	2.66	2.73	1.00	0.65	1.17	A
172/4.6	4.07	7.17	1.81	1.54	3.05	4.07	6.83	1.54	1.36	2.31	A
172/5.2	2.59	3.85	1.39	0.84	1.22	2.59	3.66	1.18	0.75	0.93	A
172/5.6	3.60	4.39	1.60	1.18	2.33	3.60	4.17	1.36	1.04	1.76	A
172/5.8	5.61	8.48	1.48	2.08	3.92	5.61	8.07	1.26	1.84	2.30	A
228/5.1	32.98	33.87	44.09	54.44	74.53	32.98	32.23	37.43	48.14	56.43	D
228/5.2	28.23	17.47	17.37	-	-	28.23	16.62	14.74	-	-	D
228/5.3	-	-	3.80	6.60	6.95	-	-	3.23	5.83	5.26	D
228/5.6	-	-	6.54	8.69	10.69	-	-	5.55	7.69	8.09	D

Dots on autoradiograph were scanned and quantitized in terms of Volume by Molecular Analyst software (Bio-Rad). These values were then adjusted according to the normalized value of the 'control dot' GAPDH. Only fragments that were subjected to further characterization are shown. '-' indicated dots that were uninterpretable due to signal masking by the neighboring dots. Expression pattern A : Constitutive expression at early hours of midazolam treatment but the level down-regulated at the 5th hour. B : Steady down-regulation. C. Transient up-regulation at 1 hour. D. Steady up-regulation. * see also Figure 3.9.

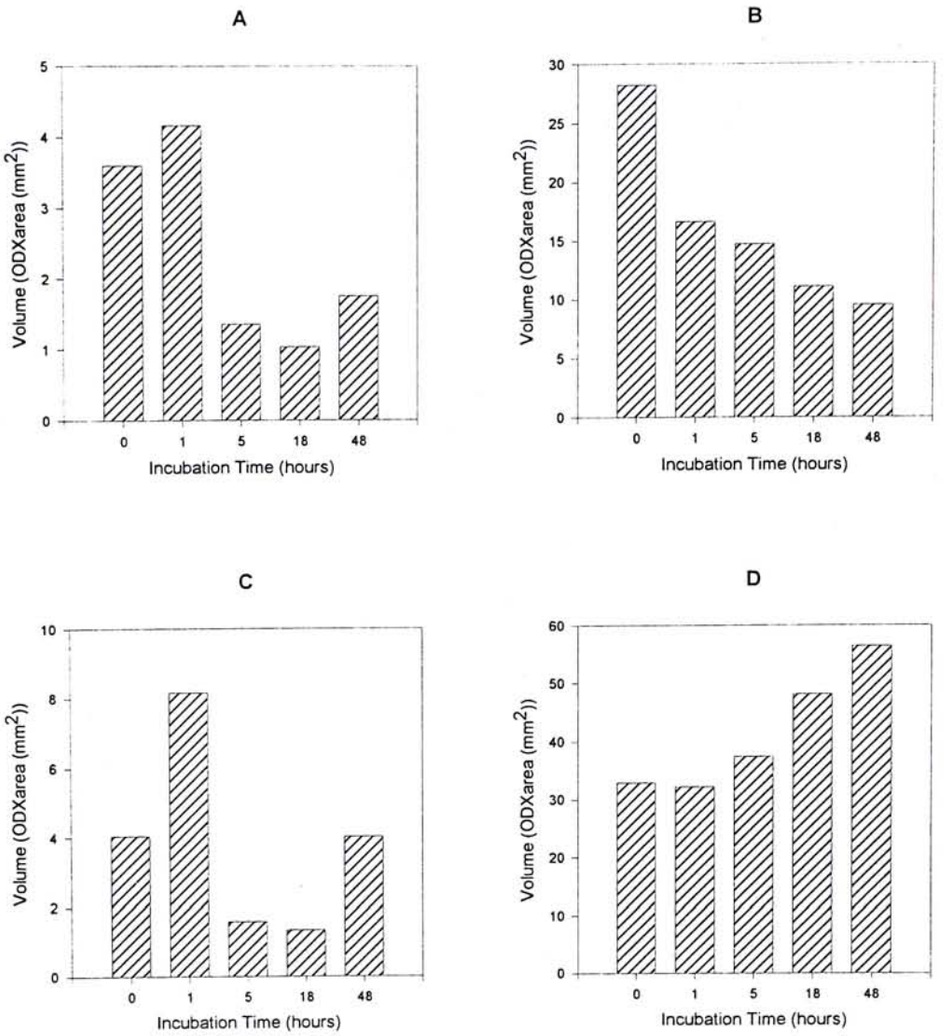


Figure 3.9 : **Expression patterns of the subcloned fragments predicted from the second round cDNA probe screening.** Four patterns were shown. A. Constitutive expression at early hours of midazolam treatment but down-regulated at the time point 5. B. Steady down-regulation. C Transient up-regulation at 1 hour. D. Steady up-regulation.

3.4.8 A comparison of the first and second screening

The expression patterns of the subcloned fragments predicted from the hybridization signals of the second screening can be traced approximately in the first screening. Some subcloned fragments like 172/2.8 and 228/5.1, 5.2, 5.3 and 5.6 showed exactly the same pattern of change in expression as their parent fragment 172/2 and 228/5 respectively (Table 3.5). Most of the subcloned fragment also followed the general trend of changes as their parent fragments (either being up-regulated or down-regulated) but variations occur at certain time points or in the degree of changes. However, there was also one subcloned fragment 147/1.4 which deviated completely from its parent fragment in expression (Table 3.5). Subcloning procedures of three parent fragments (230/5, 240/3, 228/2) were found unsuccessful.

Table 3.5. **Expression profiles of the isolated fragments predicted from the hybridization signals of the 1st and the 2nd round screening.**

Fragment	Predicted expression pattern before subcloning in the first screening	Independent subcloned fragments *	Predicted expression pattern of the subcloned fragment in the second screening
44/3	Slight up-regulation at 1 hour followed by down-reguation starting at 5 hour	44/3.1	Down-regulation starting at 5 hour
147/1	Dramatic down-regulation at 18 hour	147/1.4**	Steady up-regulation
		147/1.5 147/1.6	Steady down-regulation
172/2	Slight up-regulation at 1 hour followed by down- reguation starting at 5 hour	172/2.8	Slight up-regulation at 1 hour followed by downreguation starting at 5 hour
172/3	Dramatic down-regulation at 18 hour	172/3.1 172/3.2	Down-regulation starting at 5 hour
		172/3.8	Transient up-regulation at 1 hour followed by subsequent down-regulation
172/4	Dramatic down-regulation at 18 hour	172/4.1 172/4.6	Down-regulation starting at 5 hour
172/5	Dramatic down-regulation at 18 hour	172/5.2 172/5.6 172/5.8	Down-regulation starting at 5 hour
228/5	Steady up-regulation	228/5.1 228/5.2 228/5.3 228/5.6	Steady up-regulation
228/2	Steady up-regulation		Failure in subcloning procedure
230/5	Steady up-regulation		Failure in subcloning procedure
240/3	Dramatic down-regulation at 18 hour		Failure in subcloning procedure

* subcloned fragments derived from fragments in column 1

** subcloned fragments showing opposite expression pattern from their parent fragment.

3.5 Discussion

3.5.1 Towards the steps to isolate differentially expressed genes

RAP-PCR

Reproducibility of the RNA fingerprints has been questioned in many reports and it was found that even slight differences in concentration and quality of the same samples may result in different patterns of fingerprint (Sompayrac *et al.,* 1995; Welsh *et al.,* 1992; McClelland *et al.,* 1995). In the current investigation, it was found that two batches of total RNA from untreated JCS cells generated different patterns of polymorphic bands (Figure 3.3). This difference in banding patterns reflected that the method is highly sensitive to small variations among individual preparation. This explained partially why false positives were almost unavoidable. To minimize confusion, we stuck to the same batch of total RNA samples throughout the study.

Each lane of fingerprint generated was composed of bands both of high intensities and low intensities. Although the more abundant species of cDNAs may be amplified more frequently than the relative rare species, there were far more factors that would affect the amplification efficiencies of different species of cDNAs (even in the same tube), for instance, the sequence of the cDNA and length of the amplified sequence. Thus, no meaningful relationships between the intensities of bands and the original template concentration can be established within the same lane of fingerprint (McClelland *et al.,* 1995). Conversely, the relative concentration of the same template cDNA among different samples could be correlated roughly with the band intensities on the gel (McClelland and Welsh, 1994). In fact, this semi-quantitative nature constitutes the basics of isolating differentially expressed genes on RAP-PCR gel. Primers of different sequences but of similar size (19-24 mers) gave different patterns of bands including slightly different size range and different number of bandings. The size, number and the specificity of polymorphic bands were also affected by other factors like $MgCl_2$, dNTPs and primer concentration (Guimaraes *et al.,* 1995), the kind of enzyme used (Haag and Raman, 1994) and the annealing temperature. Some authors have tried to optimize these conditions. 4 mM $MgCl_2$ was found optimum in

the first strand cDNA synthesis (Welsh *et al.*, 1992) while 2 μM each dNTP improved the specificity during amplification (Liang and Pardee, 1992). M-MLV RNase H⁻ RT (reverse transcriptase) used in the first strand cDNA was also superior to AMV RT in generating highly reproducible bands (Shoham *et al.*, 1996) (see also Table 3.6 for comparsion of these parameters of our method with others). With regard to the use of 1.5 % Synergel in displaying the fingerprints, it appears that we enhance the speed and ease of the method at the expense of resolving power. However, our choice was justified. Using the current protocol, about 10 or more observable bands were generated. This number of polymorphic fragment was comparable to that using polyacrylmide gel (Hadman *et al.*, 1995; Welsh *et al.*, 1992; Wong and McClelland, 1994, see also Table 3.6). Although more co-migrating products were expected in our case, the problem cannot be completely solved even with gels of higher resolving power. Besides, fairly successful examples in isolating differentially expressed fragments by using 2 % or 4 % agarose (having similar resolving power of 1.5 % Synergel) have been demonstrated (Hsu *et al.*, 1993; Sokolov and Prockop, 1994).

Re-amplification

A rather simple procedure of DNA elution from the gel slices was adopted in our protocols. By boiling each gel slice (usually within 50 μl in volume) in 450 μl sterilized H_2O, the gel was melted and the DNA was released and diluted to about 10-fold; gel debris could be effectively removed by simple centrifugation. Different from some other protocol, we did not ethanol-precipiated the eluted DNA. Thus, to keep any potential inhibitors for further reactions (from the gel or previous reactions) to a minimum, dilution of templates is a 'must' for re-amplification. Using high concentration of the eluted DNA as template has proved to affect PCR leading to low yield of products (unpublished observation). Also, the eluted DNA cannot be kept for too long though eluted DNA stored for one week at -20 °C was still suitable for use. In an effort to get rid of possible non-specific contaminiated products, touchdown protocol was followed in re-amplification of isolated bands. The current thermal profile described in methods has been proved successful in eliminating non-specific products in normal PCR in our laboratory. Nevertheless, in the course of re-

amplification, several problems or observations were still encountered by us and others. Namely, some fragments (especially fragments > 1Kb) were unable to be re-amplified. This was also observed in Sokolov and Prockop (1994) and mentioned in Wong and McClelland (1994). Occasionally, single eluted fragments gave multiple bands after re-amplification as also seen in Sokolov and Prockop (1994). Besides, with the number of re-amplification increased, some contaminated bands close in size to the desired band became prominent.

cDNA probe screening

The strategy of screening a large number of isolated gene fragments recovered from RAP-PCR (or differential display) simultaneously by cDNA probes derived from RNA samples being compared has been termed by some authors as 'reverse northern blot' (Mou *et al.,* 1994; reviewed in Liang and Pardee, 1995; Livesey and Hunt, 1996). The principle of such procedure was to make DNA fragments dot-blotted (or slot-blotted) in equal amounts onto membranes in several identical copies. Each copy was then hybridized with cDNA probes generated from samples being compared. Since the same DNA fragments on each copy of membrane was indentical in amount and in excess (relative to the amount of the same sequence present in the probes), any difference in hybridization signal of the same dot on different copies of membranes should be due to the relative amount of that sequence present in the different probes and in turn in the original RNA samples. The meaning 'reverse' can be easily seen from the 'exchange' in position of the probes and the sequence to be probed with reference to the normal northern blot procedure. In addition, there were other differences between the procedures. In northern blot, different samples are hybridized in the same reaction under the same condition. In contrast, 'reverse northern blot' must be done in separate reactions and it is crucial to keep each separate reaction to have same hybridization conditions and the probes must also be normalized before used. Besides, the probes used in northern blots are single defined sequences. All the hybridizaiton signals should be due to that sequence only. On the other hand, the probes used in our screening procedures are heterogeneous. How the complexity of probes would affect the hybridization procedure is not readily foreseeable.

Nevertheless, our procedures are powerful in tackling a large amount of fragments concurrently without using excessive amount of RNA.

As mentioned earlier, re-amplification may produce some undesirable or spurious bands either due to contamination with some co-migrating species in the gel or some unknown reasons. Instead of undergoing further purification, these heterogeneous reamplifed PCR products were blotted onto the membrane and screen altogether. Since the products of the desired band usually remained dominant, hybridization signal in the screening should largely reflect the expression level of the band interested. Even if no dominant bands emerged, we were still interested in knowing the expression level of these products among the different samples. However, the first screening of those heterogenous products would render meaningless without the subcloning procedures and the second screening. Here, the subcloning procedures were not only important for further characterization of the fragment but also necessary as a kind of purification. The same PCR products used in the first screening, either apparently homogenous or heterogeneous, were used as the starting material for subcloning procedures. Despite the heterogeneity in a particular tube of PCR products, each bacterial clone contained only one type of recombinant plasmid harboring only one species of the fragments. Thus, PCR products derived from these plasmids or the corresponding bacterial lysate should be uniform and pure (Figure 3.7). Among the fragments cloned, most of them corresponded to the starting material. If the PCR products were pure enough, most of the cloned fragments would be equivalent having the same size (Figure 3.7, 44/3.1 series). On the contrary, if the input re-amplified PCR products were heterogeneous, the cloned fragments would have different sizes (Figure 3.7, 230/5.3 series). In addition, there may be some recombinant plasmids containing unrelated sequences generated somehow in the subcloning procedures in both cases. Thus, to encompass all the DNA species present or more importantly to isolate the species that were responsible for the differential signals among different samples during the first screening, enough bacterial colonies must be picked. Of course, the actual number of recombinant clones that should be picked were justified with the time and effort required. No matter how many clones that we picked, we were never completely sure if all the fragments present on the interested dots selected in the first screening were included. As a result, a second

screening was introduced aiming at discerning fragments which still showed differential expression among different time point of treated JCS cell sample. Since the PCR fragments on the blot were relatively pure in the second screen, the hybridization signal should more truly represent the expression profile of the fragments. In some cases, the expression profile of some fragments predicted in the second screening can be traced in the first screening. However, even if we failed to establish a relationship between the two screenings, the results of the second screening can still be interpreted independently. Two screening procedures were not redundant although they required the same techniques. The first screening was designed mainly to eliminate 'false-positives' generated by RAP-PCR while the second screening was set to remove those contaminiated and/or co-migrating bands on the RAP-PCR gel and spurious bands produced during the subcloning steps. Only the most robust fragments 'surviving' two rounds of screening would be further characterized. In spite of the rationale behind, one may argue that such method may overlook certain differentially expressed fragments. As we aimed at a large scale production of differentially amplified bands observable on RAP-PCR gels finally, it is more beneficial and practical to design a screening system that are reasonably strict that favored no 'false-in' elements (though'false-out' may occur). As a matter of fact, the 'false-out' problem commonly exists in other methods for seeking differentially expressed genes (e.g. differential screening and subtractive hybridization).

3.5.2 Expression profile predicted at different stages of the procedure

Almost all kinds of expression pattern possible for the isolated bands (genes) as predicted from the gels of RAP-PCR were shown. Expression only at early hours typical of some transcription factors (e.g. c-*myc*), steady and gradual up-regulation or down-regulation resembling some integrin genes (e.g. CD11c) and biphasic regulation like SCL gene were observed (Murrell *et al.*, 1995; Noti and Reinemann, 1995). There were also some differentially amplified bands that occurred at only one particular time point (see Figure 3.3 for examples). In view of false positives and non-reproducible bands on RAP-PCR gels, the true expression patterns of these isolated bands (genes) need further elucidation.

After the first screening, the expression patterns of the potential fragments (genes) that may have differential expression during midazolam-induced differentiation were narrowed down into three types (Figure 3.6). Expression profile type A and type B described a dramatic decrease in hybridization signal at 5 and 18 hour respectively while expression profile type C depicted a relatively gradual and moderate up-regulation. Fragments (genes) showing expression at only one particular time point were not observed in this screening. This intense decrease in the types of expected expression profile of the fragments (genes) further stressed that a high proportion of false positives and/or non-reproducible bands were generated during the RAP-PCR procedure.

In the second screening more expression patterns (four) were found. (Figure 3.9). While two described down-regulation, two illustrated up-regulation. On close examination, the expression pattern of individual subcloned fragments (genes) on the second screening can be traced in the first screening (Table 3.5). However, variations in the degree of change and hybridization signal at a certain time point were observed. Such differences can be accounted for, by the fact that subcloned fragments were purer (in terms of no. of DNA species) than their parent fragment (re-amplified products). Hybridization signal from each dot in the first screening could have been contributed by several different DNA sequences showing the same type of expression profiles. This was exemplified by the independent subcloned fragment derived from

the same parent fragment (re-amplified products) 172/4, 172/5 and 228/5 (Table 3.5). Conversely, there were parent re-amplified products composed of DNA species having different or even opposite expression profiles like that shown by 147/1.4, 1.5 and 1.6 (Table 3.5). This demonstrated the heterogeneity of the original re-amplified products studied in the first screening. Besides the purity of the fragments under investigation, the variation in probe preparation and method of data processing may also affect the relative final hybridization value (volume) of different time points. As mentioned, the cDNA probes used were very complex. Although every effort was made to keep the preparation of probes of different time points as similar as possible, it is conceivable that small variations of each step in the multi-step procedure would accumulate. In addition, the adjustment of hybridization values of each dot (according to the slopes between 'GAPDH' dots on membranes of different time point) (Table 3.3, 3.4) may not be appropriate as the signal response of the X-ray film was not linear, especially for the highest exposure (Wygant and Nelson, 1995).

3.5.3 Representation of the total mRNA in the cell

It was suggested that most of the mRNAs are represented as one band in RAP-PCR. In this report, each lane of fingerprint had 1-12 bands and most of the fingerprints gave 6-9 bands per lane. Thus, each lane of fingerprint generated by a single primer represented 6-9 messages. In a typical eukaryotic cell, there is approximately 1 pg of mRNA equivalent to about 10^6 molecules, transcribed from about 15,000 different genes (i.e. a total complexity of 15,000) (reviewed in Sargent, 1987). If each of the single primer in our experiment produced 9 bands and a total of 44 primers were used, 396 messages were produced. That is, 396/15,000 X 100% = 2.64 % of the total messages were represented. Without considering the abundance of a particular message in the total mRNA, we calculate the probability of missing a message $P(0) = e^{-\mu}$. To detect a particular message with 95% chance i.e. P(0)= 0.05 without taking into consideration the abundance of the message in the total mRNA, μ would be equal to 3. That means the number of bands that is three times of the total complexity (15,000 X 3) must be obtained. If on average 9 bands/messages could be sampled per single primer, the whole complexity of the cell could be represented using (15,000/9) X 3 = 5000 primers at a 95 % confidence level. If 4 sets of RAP-PCR reaction each using one primer could be displayed in a gel, a total of 1250 gels would be needed (McClelland and Welsh, 1994).

This calculation illustrated that it is almost impossible to obtain a particular message, especially that of the rare species, if our sampling is as low as less than 3% of the total message. However, it is expected that many genes are involved in the differentiation induced by midazolam and we may be able to encounter some of these genes.

3.5.4 Comparison of the original and modified protocol of RAP-PCR

As an evaluation of our methods, we compare our protocol with the original protocol first devised by Welsh *et al.* (1992). The two protocols are the same in principle although there are some variations (Table 3.6).

Total RNA was found sufficient in general for RAP-PCR and used in both methods as starting materials. Total cellular RNA and poly(A)$^+$ RNA used with anchored oligo-dT primers generated essentially the same banding pattern in differential display (Liang *et al.,* 1993). Thus, different from the protocol used in Welsh *et al.* (1992), we tried using oligo-dT primers ranging from 12-18 bases instead of arbitrarily-chosen primers (20-mers) to make the first strand cDNA. Using arbritrary primers in the first strand cDNA synthesis had a potential problem that the more abundant rRNAs or even the tRNAs may be amplified. Although most of these RNAs are not generally differentially expressed (Wong and McClelland, 1994) affecting the interpretations of the fingerprints, a prior selection of mRNAs by oligo-dT would diminish these abundant sequences from competing for primers and substrates with the messenger sequence. On the other hand, using arbritrary primers for the first strand cDNA synthesis increases the chance of obtaining product sequence away from the 3'end untranslated region. In the second strand cDNA synthesis and subsequent amplification, arbitrarily-chosen primers (about 20 bases) were used in both cases. During the amplification, however, one long low stringency cycle (each step lasting for 5 minutes) was used in the original protocol instead of 5 normal length (each step lasting for 1 minute) low stingency cycles in our methods. A total of 30 or 40 (in later protocol by the same authors) of high stringency cycles were performed in the orginial protocol but 45 high stringency cycles were done in ours. Using our protocol, the number of low stringency cycles (from 2-10) did not alter the pattern of fingerprints significantly (unpublished observation) and the mean number 5 was taken to secure sufficient priming. Alternatively, extended period of each amplification step as described in Welsh *et al.* (1992) for a lower number of cycle(s) can be used. The fragments generated during the low stingency cycle(s) were further fixed by subsequent high stringency cycles. Increasing the number of high stringency cycle in

this report was necessary to compensate for the relatively low sensitivity of ethidium bromide staining. However, this increase did not seem to affect the identification of differentially amplified fragments across the samples. As explained previously in McClelland *et al.* (1995), the ratio of starting materials between samples is maintained even the cycle number is sufficient to lead the amplification to the plateau phase.

By comparing some statistics of the fingerprints, appreciable differences were found between the two protocols. On a high resolution 4-6 % polyacrylamide gel in the original protocol, the number of resolved bands were 2-4 fold more than what we could be obtained in the Synergel per fingerprint (Table 3.6). Nevertheless, the sizes of the polymorphic bands that could be generated and resolved in Synergel were significantly larger (about 2 to 3 -fold) than those observed in Welsh *et al.* (1992) (Table 3.6). Such observations in the fingerprints could be explained by the amplification reacion itself. Since our method have selected for mRNAs during 1st strand cDNA synthesis, non-mRNAs sequence amplification should be mininimized and less bandings may thus be produced. In addition, the relatively more efficiently amplified small bands (a few hundred base pairs) may be able to give way to the longer sequence (larger than 1Kb) under our conditions. The resolution and the size range that the gel could resolve may also contribute to the explanation. 4-6 % polyacrylmide gel is optimized for fragment size below ~600 bp while 1.5 % Synergel are suitable for fragments of ~0.1-5K bp. Thus, larger fragments may not be easily recognized in the polyacrylamide gel. On the other hand, the resolving power of 1.5 % Synergel was much lower, bands of very similar size (within a few tenths of base pairs) might not be readily separated and gave an apparently less number of fragments in the fingerprints. Our method has also abandoned the use of radioactive label in visualizing the polymorphic bands but relied on ethidium bromide staining. Boiling the gel pieces for 5 minutes was also found sufficient for Synergel despite the long elution period employed in the original protocol for polyacrymide gel (see Table 3.6 for details). Since the polymorphic bands across the different samples could be identified immediately under UV excitation (without the need of developing autoradiographs) and recovered easily, our method saved time and reduced potential hazard.

In avoiding false positives, different strategies were employed. In Welsh *et al.* (1992), efforts were made to prevent false positives caused by intra-sample variations. In our protocol, two rounds of cDNA probe screening or known by others as 'reverse northern blotting' (Mou *et al.,* 1994; reviewed in Liang and Pardee, 1995; Livesey and Hunt, 1996) were used.

Table 3.6. **A comparison of the original RAP-PCR protocol and our method.**

	RAP-PCR by Welsh *et al.* (1992)	RAP-PCR in this report
Starting materials	Total RNA	Total RNA
Primers in 1st strand cDNA synthesis	One arbitrarily-chosen primer	Oligo dT
$MgCl_2$ concentration in 1st strand cDNA synthesis	4 mM	3 mM
Reverse transcriptase	M-MLV (Strategene)	M-MLV(GIBCO)
Primers in 2nd strand cDNA synthesis	One arbitrarily-chosen primer (same as the one in 1st cDNA synthesis)	One arbitrarily-chosen primer
dNTPs concentration in 2nd cDNA synthesis	50 μM each dNTP	200 μM each dNTP
Length of primers	20 bases	19-24 bases
Amplification enzyme	AmpliTaq, Cetus	Thermoprime$^+$ DNA polymerase, AB
Low stringency cycle	One cycle, each step for 5 minutes	Five cycles, each step for 1 minute
High stringency cycle	30 cycles (denaturation, primer annealing for 1 minute, extension for 2 minutes)	45 cycles (each step for 1 minute)
Fragment size fractionation	4 % - 6 % TBE polyacrylamide gel	1.5 % TAE synergel
Visualization of polymorphism	α–[^{32}P]-dCTP label	Ethidium bromide staining
Isolation of differntially amplified PCR product	Gel pieces eluted at room temperature for 1 hr into 50 μl of TE	Gel pieces boiled in 450 μl of H_2O for 5 minutes
No. of resolved bands per fingerprint	10-20 (Welsh *et al.*, 1992) 10-40 (Perucho *et al.*, 1995) 10-50 (Welsh *et al.*, 1995)	1-12
Size distribution of the resolved bands	~200-600 ~50-1000 (McClelland and Welsh, 1994)	~200-2000
Strategies to reduce the occurrence of false positives	Comparing polymorphic bands generated from different concentrations of the same sample Single low stringency cycle to prevent genomic DNA contamination	Two rounds of cDNA probes screening Re-amplification by touchdown PCR to remove false positives caused by infrequent imperfect primer annealing

3.5.5 Advantages of the modified protocol and further refinements

As a summary of what we have discussed in previous sections, several strong ideas of our procedures are highlighted here. One of the most powerful refinements in our protocol was to adopt two rounds of cDNA probe screening (or reverse northern blotting). The strategy allowed a preliminary estimation of expression of the isolated fragments and should eliminate a large number of 'false positives' and fragments having no detectable expression, especially in the first round screening. The second round screening further took away unrelated sequence introduced in the subcloning procedures. The importance of performing selection proceudure after subcloning was also noted by others (Callard *et al.,* 1994; Zhao *et al.,* 1996). Different from our procedures, they used the PCR fragments before subcloning to probe the recombinant plasmids obtained from several independent clones. Another important improvement of this method was that we selected also those co-migrating or contaminating bands instead of avoiding them. The reasons is obvious. First, it is practically difficult to remove or separate these bands completely. Second, for co-migrating species, it is unable to tell which DNA species within 'one' band are responsible for the change in band intensities across the time points. Thus, it is certainly 'safer' to include all possible fragments. However, such strategies would not be a success without an effective (quick and easy to perform) selection system that could deal with enormous amount of fragments at the same time. The use of primers in our method also deviated from a typical RAP-PCR protocol. With the use of oligo-dT primers in the first strand cDNA synthesis, we avoided sequences other than messenger RNA. At the same time, we would not miss out sequence outside 3'end region completely having the use of arbitrary primers in the second strand cDNA synthesis (Haag and Raman, 1994). The use of radioactive labels have been minimized in our methods and high-voltage polyacrylmide gel were replaced with easily prepared 1.5 % Synergel. Our system represented a safer, quicker and simpler method.

The current protocol and selection system are not intended for isolating all the mRNA species (section 3.5.3) or picking a certain sequence but are good for seeking a group of sequence fragments that are differentially expressed among the samples

being studied. RAP-PCR has been said to under-represent the rare mRNA species (McClelland and Welsh, 1994). Thus, it is also important to know if the fragments isolated from our procedures and screening systems came from the rare population. Also, it may be beneficial to optimize the conditions for the first strand cDNA synthesis and RAP-PCR. Efforts have been made to optimize conditions like $MgCl_2$ concentration in the first strand cDNA synthesis (Welsh *et al.,* 1992), $MgCl_2$ concentration relative to dNTPs concentration (Bauer *et al.,* 1993), dNTPs concentration (Guimaraes *et al.,* 1995), types and sequences of the primers (Bauer *et al.,* 1993; Haag and Raman, 1994; Zhao *et al.,* 1995), brands or batches of reverse transcriptase and thermal enzymes (Haag and Raman, 1994), temperature and time for primer annealing (Bauer *et al.,* 1993; Zhao *et al.,* 1995). It seemed that it became absolutely necessary to optimize conditions for the production of robust and reproducible bands on the RAP-PCR gel after we have employed the cDNA probe screening system. Nevertheless, optimized conditions allowed the maxmium utilization of each components in the reaction and increased the representation of mRNA species (bands) in the cell per primer used (section 3.5.3). As a result, the chance of obtaining differentially expressed genes, especially those belonging to the rare species would be enhanced.

In conclusion, to make our current system a success, it is important to generate enough amount of polymorphic fragments during the step of RAP-PCR. Besides, reducing the times of reampification may be helpful. With a good planning and estimation of the experiment, enough PCR products can be amplified at one time for both subcloning and selection procedures. Keeping the conditions the same for probe preparation from different samples is also very crucial. Finally, it is necessary to mention that the selection system using cDNA made from total RNA of different samples cannot be a final proof of the expression profile of the fragments under investigation (and their corresponding genes) because of the heterogeneity of the probes and the complex hybridization kinetics. Further characterizations of the isolated fragments after the two round screening are indispensable.

Chapter Four Characterization of the Putative Differentially Expressed Genes in Midazolam-induced JCS Cells

4.1 Introduction

Two types of characterization are essential for the cloned and selected gene fragments obtained as described in the last Chapter. One of these is to determine whether the gene fragments belonged to a known or a novel gene. This can be accomplished by sequencing followed by homology search against the Genbank. More importantly, it is necessary to prove (or to confirm) the isolated gene fragments are indeed differentially expressed during the midazolam-induced JCS cell differentiation. Several strategies can be employed for confirmation and RT-PCR represents one of the most convenient and sensitive methods.

4.1.1 DNA sequencing

Sequencing of nucleic acid can be traced back to 1960s after the double-helix structure of DNA was discovered in 1953. The major work before 1965 was concentrated on small and relatively available tRNAs. They are broken down into small pieces of a few nucleotides long and each of them was sequenced using chromatographic methods. The first sequence of an intact molecule was an 80-base yeast tRNA published in 1965 (reviewed in Chen, 1994).

DNA sequencing was only possible after the discovery of restriction enzyme and DNA polymerase around 1970. In the mid-1970s, two independent DNA sequencing techniques were developed and increased greatly the rate of sequencing. One of them was the chain termination method invented by Sanger *et al.* (1977). The method was successfully used to sequence the 16.5 kb human mitochondria genome in 1981. Another one was the chemical degradation method developed by Maxam and Gilbert (1977). It was then successfully applied to the analysis of the 40kb T7

bacteriophage sequence. Since then, sequencing data have become more and more important in understanding the biological systems (reviewed in Chen, 1994).

The chain termination method was also known as dideoxy sequencing. The DNA strand to be sequenced was first annealed by a primer, usually a synthetic oligonucleotide to make a complementary strand catalyzed by a DNA polymerase. The method employed the dideoxynucleotides, which lacks the hydroxyl group at the 3' position of the sugar residue, and thus terminates the elongation reaction. Since the modified nucleotides incorporate as efficient as the normal nucleotide into the growing strand, they can compete with the normal substrate in the DNA polymerization process. For each sequencing reaction, four independent DNA synthesis reactions are carried out in parallel, each containing a small amount of either dideoxyadenoside, dideoxycytoside, dideoxyguanoside or dideoxythymide. The random and low level incorporation of a specific dideoxynucleotide gives a mixture of DNA fragments of different lengths ending with the respective dideoxynucleotides. The reactions can then be chased out to longer fragments with increased concentrations of normal analogue to ensure all the fragments stopped at the specific dideoxynucleotide. The fragments can be visualized on a polyacrylmide gel by replacing one of the normal nucleotides with a radioactive-labeled counterpart in the reactions or by using an end-labeled primer.

There are still several variations that one can follow for chain termination method. For instance, different DNA polymerases can be used like Klenow fragment of *E. coli* of DNA polymerase I, reverse transcriptase, bacteriophage T7 DNA polymerase without 3' to 5' exonuclease activity (Sequenase and Sequenase version 2.0) and thermostable DNA polymerase (e.g. *Taq* polymerase). Klenow fragment is only good for DNA within 250 bases while reverse transcriptase is only occasionally employed to resolve problems caused by homopolymeric A/T or G/C. Sequenase and thermal polymerase are more commonly used nowadays with good processivity and high rate of polymerization. For cycle sequencing, thermal enzyme is the only choice (Sambrook *et al.*, 1989).

The template for sequencing can also be treated in several ways. The re-amplified PCR products from RAP-PCR fragments can be cloned into phagemid vector to generate single-stranded DNA or into plasmid vector for double-stranded sequencing. Alternatively, the raw products can be directly sequenced or cycle-sequenced. Single-stranded sequencing usually yield good result but double-stranded templates are more convenient and simple to prepare. Sequencing without cloning procedures needs additionally synthesized primers. Cycle sequencing needed considerably less amount of templates but additional purification steps to remove excess primers and dNTPs are necessary before the sequencing reaction can be performed (Sambrook *et al.,* 1989).

The chemical degradation method (Maxam and Gilbert, 1977), instead of synthesizing new DNA fragments, breaks down the terminally-labeled starting material into fragments. Special chemicals are added to four separate reaction mixtures so that the DNA molecules are cleaved preferentially at guanines, at adenines and guanines equally, at cytosines and thymines equally, and at cytosine alone. The reactions are done at limiting conditions and only one cleavage is made on each molecule on average. When the fragmented products are resolved by polyacrylmide gel electrophoresis, the length of the product will identify the positions of a particular base. Although the chain termination method is much quicker and easier to perform nowadays, the chemical method are still used by some scientists. The advantages of chemical method include (1) It is not affected by long runs of the same nucleotides like poly C. (2) Synthetic oligonucleotide can also be sequenced directly without cloning procedures. (3) It also allows studies like analyzing DNA modification e.g. methylation, examination of DNA secondary structure and protein/DNA interaction by chemical protection/modification interference experiments (Barker, 1989).

4.1.2 Automated DNA sequencing and analysis

Automation

The application of automation to DNA sequencing and analysis involves a tremendous range of techniques and strategies. The first software tools for interpreting DNA sequence data were developed in the late 1970s and early 1980s. Software for assembling sequence fragments into contigs began to appear in the mid-1980s. Since 1985, several automated sequencers were developed with a view to automate gel electrophoresis, raw data acquisition, and base-calling (Smith *et al.*, 1986). Automated DNA sequencers based on fluorescence detection of electrophoretically separated fragments were available commercially in 1987 and in the same year the first gene determined by automated sequencing method was published (reviewed in Chen, 1994). Other aspects of automation have also been developed. Computer-operated robotics workstations and more sophisticated softwares have been applied to prepare sample, to handle the sequencing reactions and to assemble data (reviewed in Bankier, 1993; Hunkapiller *et al.*, 1991). Commercial sequencing reaction robots with automated cycle sequencing was available in 1991 and sample preparation robots were introduced in 1992 (reviewed in Chen, 1994). Integrated systems that carry out the entire DNA sequence analysis process without manual intervention are vigorously discussed and developed by several groups.

Automated sequencers and their dye chemistry

There are currently two different instrumental designs of automated sequencers. One of them utilizes densitometric film scanner digitizing images of sequence ladders from film or autoradiograms, exposed and developed after fixed periods of electrophoresis. The other systems employed fixed or scanning detectors which can monitor the electrophoretic transport of labeled fragments through the gel, giving digital signals of the sequencing ladders in real time.

According to the number of fluorescent dyes used, the commercially available automated sequencers can be classified into two types. The first type uses single-label, four-lane separation and has 10 to 12 channel capacity (Brumbaugh *et al.,* 1988) while the second type applies four-label, single lane separation, and currently has 36-capacity per run (Connell *et al.,* 1987). The commercial brand belonging to the first type include Pharmacia ALF, LI-COR 4000 and Millipore Basestation while ABI 373A sequencer is classified as the second type. The fluorescent dye can also be labeled either at the primers (dye-primers) for four-tube reaction but single lane loading or at the terminators (dye-terminators) for single tube reaction and single loading (reviewed in Chen, 1994).

Automated Laser Fluorescent (ALF) system

The system generally utilizes chain termination method with the application of fluorescent label. Single type fluorescent label can be attached to the sequencing primer and excited by a laser beam. The laser beam of the ALF is directed through the gel across its width. The emitted fluorescence is collected by 40 fixed detectors (one per lane) near the bottom of the gel in real time as the fragments migrate during an electrophoresis. Simultaneous recording of ten sequences can be performed in about 6 hours (relative short compared to other systems) (Figure 4.1). Active temperature control of the gel is provided for a more consistent electrophoretic separation. The optical system of ALF is optimized for a single dye and the detection is relatively sensitive for all four lanes. Also, the labeling chemistry can be used with conventional automated DNA synthesis as well as chemical DNA sequencing. Lengthy purification is unnecessary even with crude labeled primers (Ansorge *et al.,* 1993; Hunkapiller *et al.,* 1991).

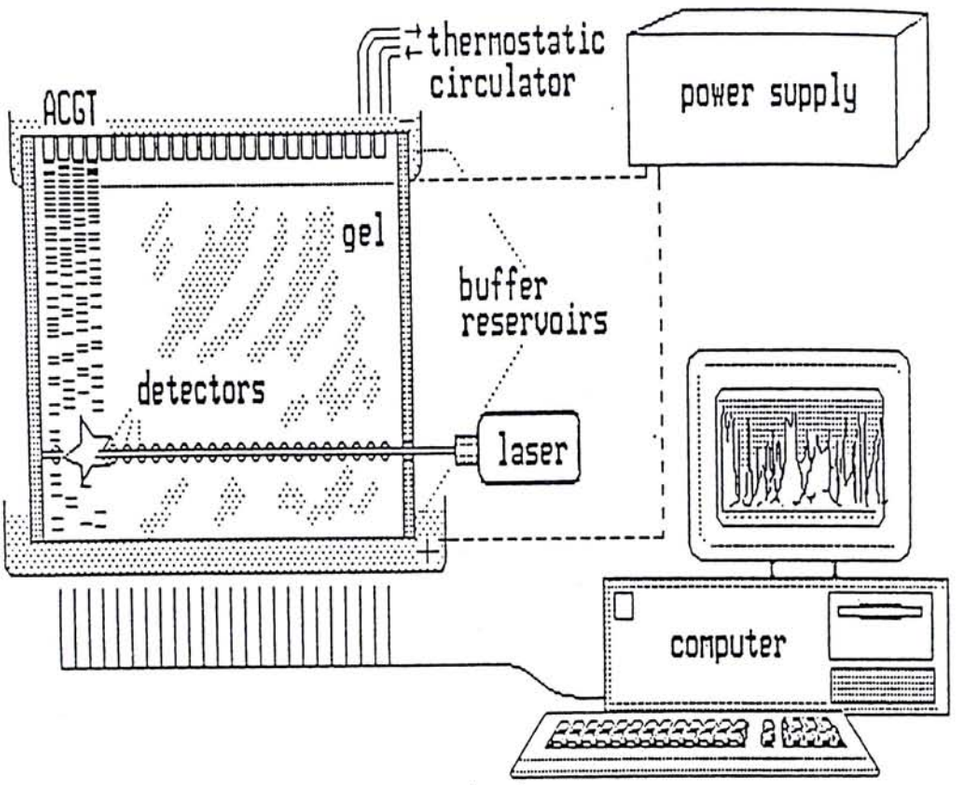


Figure 4.1. **Automatic Laser Fluorescent (ALF) system.** A water thermostated polyacrylamide gel is used for fragment separation. A laser beam is projected across the entire width of the gel at a fixed distance from the top of the gel. As soon as fluorescently labeled fragments enter the beam, they become excited and emit fluorescent light that is continuously measured by the optical detector for each single lane. A maximum of ten sequence (4 lane per sequence) can be performed at one time. Off line evaluation allows automated sequence determination. (Reproduced from Ansorge *et al.*, 1993)

4.1.3 Genbank and BLAST homology search

Genbank

Genbank, a trademark of the U.S. Department of Health and Human Services, is a comprehensive repository of sequence data and associated annotation sponsored by the U.S. National Institutes of Health (NIH). It is built, maintained and distributed by the National Centre for Biotechnology Information (NCBI). Genbank is also collaborated with the EMBL data library sponsored by the European Molecular Biology Laboratory and the DNA Data Bank of Japan (DDBJ) sponsored by National Institute of Genetics, Mishima, Japan. These three major DNA databases exchange sequence information and submissions on a daily basis and very often, the term Genbank are used in a boarder term to include the databases Genbank, EMBL and DDBJ.

GenBank has been expanded to encompass all known sequences of 20 nucleotides long including all published DNA sequences since early 1990. The data are distributed six times a year on CD-ROM and by anonymous FTP (file transfer protocol) on the internet. There are also RETRIEVE and BLAST server for users to retrieve database records and perform sequence similarity searches respectively.

Genbank is not only a DNA but also a protein database. It maintains all known protein sequences together with all published peptide sequences of at least six amino acid long. It is also combined with a subset of the MEDLINE bibliographic database, the Molecular Sequence Data Subset, retrievable through Entrez system (Ouellette and Boguski, 1995).

Blast sequence search

BLAST, stands for 'Basic Local Alignment Search Tool' (Altschul *et al.*, 1990), is a series of programs designed for database similarity searching. A query sequence can be fed in and the program will compare it with all known sequences in a

database and output a ranked database sequence list according to the closeness with the query sequence. The BLAST programs include 'blastp' which compares amino acid query sequence against a protein sequence database. 'blastn', on the other hand, compares a tested nucleotide sequence against a nucleotide sequence database. Other variations are 'blastx' which compares the six-frame conceptual translation products of a fed-in nucleotide sequence against a protein sequence database and 'tblastn' which compares a protein query sequence against a nucleotide sequence database dynamically translated in all six reading frames. 'tblastx' compares the six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence database.

Sequencing database searching programs can be exhaustive or can take shortcuts to reduce the time needed. The time taken to compare two sequences is proportional to the product of their lengths whereas the time taken to locate identities between sequences is proportional to the sum of their lengths.

There are three main ways to access BLAST search. The first one is through E-mail at the server address (blast@ncbi.nlm.nih.gov). The service is also available at a WWW interface (http:\\www.ncbi.nlm.nih.gov) or as an Internet service using a command-line interface. E-mail service is convenient and well-suited for occasional search. The WWW interface allows the use of 'copy and paste' sequences for searching. The command-line interface is the most powerful way and is easy to incorporate into automated systems for high-throughput analysis (Recipon *et al.*, 1995).

In this report, only BLASTN program will be used. There are several outputs for BLASTN algorithm. The following is a summary of their definition (Altschul *et al.*, 1990; Recipon *et al.*, 1995):

Table 4.1. **A summary of the output parameters in a BLASTN search.**

Parameters	Definitions
B	The maximum number of database sequences for which high-scoring segment pairs will be reported.
Bits	Raw score conversion by Lambda value
E (Expectation Threshold)	The upper limit of the expected frequency of chance occurrence of a HSP within the context of the entire database search.
E2	The expected number of HSPs that will be found in comparing two sequences that each has the same length.
Effective length	The real length minus the expected length of the HSP. The expected length is calculated from its score
H	The relative entropy of the target and background residue frequencies.
High Score	The best ungapped local alignment score, based on the scoring matrix used
HSP(High-scoring Segment Pair)	It consists of two sequence fragments (one from the query sequence, one from the database) of arbitrary but equal length. Their alignment is locally maximal meeting/exceeding a threshold or cutoff score.
Identities	The number and fraction of total residues in the HSP which are identical
K	Karlin-Altschul parameter
Lambda	The expected increase in reliability of an alignment associated with a unit increase in alignment increase.
MSP (Maximal-scoring Segment Pair)	Highest-scoring of all possible segment pairs from two sequences.
N	The number of HSPs in the sets that resulted in the lowest p-value.
P	The probability that the match could have occurred by chance
P(N) (Smallest Sum Probability)	The lowest P-value ascribed to any set of HSPs for each database sequence
Positives	The number and fraction of residues for which the alignment scores have positive values.
S	The score at which a single HSP would by itself satisfies the significant threshold E.
S2	The score expected for the MSP between two sequences.
T	Neighborhood word score threshold
V	The maximum number of database sequence for which one-line descriptions will be reported.
W	Short word length that satisfies some positive-valued threshold score T
X	Positive integer representing the maximum permissible decay of the accumulating segment score during word hit extension

4.1.4 Internal primer design for RT-PCR

Several methods have been employed to confirm expression pattern of the gene fragments isolated in either RAP-PCR or DDRT-PCR including the conventional northern blotting (Ralph *et al.,* 1993), RNase protection assays, nuclear run-on assays (Bauer *et al.,* 1994) and reverse-transcription polymerase chain reaction (RT-PCR) (Momiyama *et al.,* 1995). The advantage of using RT-PCR to study gene expression over other methods have been discussed in section 2.1.4. In contrast to other methods where sequencing can be done after the gene fragments have been confirmed to be differentially expressed in the model under study, sequencing must be done in advance for selection/design of primers used in RT-PCR.

The choice of primers is one of the most important factors in determining the quality of a polymerase chain reaction. Generally speaking, the more information about the DNA template is available, the better the primers for the reaction could be designed. In selecting PCR primers, there are some essential parameters that needed to be considered. First, the primer selected can form stable duplex with the specific site on the target DNA. Second, the primer should not have duplex formation with another molecules. Third, the primer should not prime on any other target site (false priming). To examine the duplex stability, the free energy of duplex formation ($\Delta G = \Delta H - T\Delta S$) can be calculated where T, ΔH and ΔS are the temperature in K, enthalpy and entropy of duplex formation respectively. To minimize primer-dimer artifacts which reduce product yield, the PCR primers selected should be free from complementarity especially at the 3' termini. Self-complementarity leading to internal primer extension should also be noted; intra-molecular duplexes with negative ΔG should generally be avoided. To eliminate false priming, primer structure with a stable 5' termini but an unstable 3' termini was effective (Rychlik, 1993). This is because the priming on non-target DNA site at the 3' end is inefficient to initiate an extension unless stable duplex could be formed at the 5' end and the central part of the primer, as in the case of the target DNA.

Other small 'tricks' of selecting suitable primers include avoiding 3' T. As T is the least discriminating nucleotide at the 3' position, primers with a 3' T have greater tolerance of a mismatch. It is also advisable for each primer to have at least one A or T within the 3' most triplet to prevent mismatch tolerance of primers with consecutive Gs or Cs. The melting temperature of the primer pair in a PCR should be roughly the same ($\pm$ 2 $^{\circ}$ C). The size of the amplified products should at best fall in the range of 100-600 base pairs for efficient amplification (Sharrock, 1994).

Besides general considerations of primer design, attention should also be undertaken for gene fragments amplified by RAP-PCR. Up to two to four mismatches were found in comparison to the original cDNA towards the 5' end of 10-mer arbitrary primers (Liang *et al.,* 1995). Thus, to ensure specific primer annealing in RT-PCR done on total RNA, the region of the arbitrary primer sequence or at least those towards the 5' end should be avoided in primer selection.

Since a lot of parameters have to be taken into account and many calculations need to be performed in designing primers, computer-aided PCR primer design like using programme OLIGO (National Biosciences) which calculates the annealing temperature of PCR reaction, taking into consideration the stability of the PCR products as well as the primer Tm values has greatly facilitated the process. Finally, it is also worth mentioning that PCR primer sequence is only one of the determining factors in generating clean and specific products. Other factors like substrate complexity, the length of the product, the annealing temperature, the cycle profile, the buffer composition and the enzyme also affect the reaction. Varying some of these conditions may help improving the specificity of the primer (see also touchdown PCR described in section 3.1.3).

After suitable primers are designed, the expression pattern of the genes represented by the isolated gene fragments can be determined during the midazolam-induced JCS cell differentiation. In addition, the primers can be readily applied to expression profile studies on other RNA samples. It is our interest to examine the expression pattern of the isolated genes during biochanin A-induced JCS cell differentiation. Biochanin A, as opposed to midazolam, induced JCS cells to produce

monocytes only; studying the expression profiles of the isolated genes during the biochanin A-induced differentiation, when compared to those in midazolam-induced cells, may allow us to speculate roles of these genes on lineage commitment.

4.1.5 Genes involved in both myeloid cell differentiation and embryonic development

To extend our understanding towards the isolated gene fragments, we suggest to study at the same time the expression of these gene fragments at different stages of the mouse embryos. There are at least two reasons for initiating such studies. First, leukemic cells are abnormal cells; not all of their gene expressions are comparable to normal tissues. Consequently, by studying the expression of the isolated gene fragments (or the corresponding genes) in normal tissues like that in the embryos may allow us to give an account on whether the expression is specific to leukemia cells or is occurred in normal tissues in general. Second, examining the expression profiles of these gene fragments during embryonic development gives us more suggestions on the possible function of the genes (especially for those previously unknown genes). Two indications proposed that genes responsible for myeloid cell differentiation may also be developmentally regulated. (1) Embryonic development involved considerable highly ordered differentiation process. Thus, myeloid cell differentiation and embryonic development may have common machinery that dictates cell proliferation and/or differentiation (Pavelic *et al.*, 1991). Ubiquitously expressed genes involved in myeloid cell differentiation (e.g. c-*myc* and c-*fos*) belonged to this group. Growth factors (e.g. FGF, TGF-β, LIF, SCF), their receptors (FGF-R) and transcription factors (e.g. c-*myb*) are all possible common genes involved in both processes. (2) Unlike the differentiation-related genes, there are other genes that involved in myeloid cell differentiation but are expressed in embryo carrying out entirely different gene function (e.g. *Hox* family). Hence, it is certainly fruitful to conduct expression profile studies of the isolated genes on embryonic development.

The time points chosen to be studied represent different stages of development : 7.5 dpc (gastrulation), 9.5 dpc (early organogenesis), 11.5 dpc (organogenesis), 15.5 and 17.5 dpc (fetal growth and development). Distinct features at 7.5 dpc include late primitive streak, appearance of allantois, blood islands in visceral yolk sac, foregut pocket and neural plate. At 9.5 dpc, 21-29 somites are seen and forelimb buds formed at somites 8-12. There are also common ventricle, atrium and fused dorsal aortae. Lung primordia, pancreas evagination, and vitelline ducts also formed. Posterior

neuropore closes; optic and otic vesicles are observed. At 11.5 dpc, the embryo is 6-7 mm in size with forefoot plates. There are partitioned atriums and unpaired ventricle. Bucconasal membrane and lens vesicles are also detached and ureteric buds formed. At 15.5 dpc, the embryo grows larger to 12-14 mm in size. The coronary vessels formed and palatine processes fused. At 17.5 dpc, the embryo is 17-20 mm large. The alveolar ducts of lung develop and the ciliary body is delineated (reviewed in Hogan *et al.*, 1994).

During the early post-implantation period of the embryo (5-10 dpc), many genes controlling differentiation and organogenesis are gradually brought into play. Some of them have already shown their involvement in myeloid cells. For instance, c-*kit* is essential for the formation of myeloid progenitors and CFU-S activity in adult (reviewed in Paulson and Bernstein, 1995). In embryo, however, SCF and c-*kit* affect not only hematopoietic tissues but also primordial germ cells and neural crest cells (reviewed in Hogan *et al.*, 1994). LIF was first described to induce myeloid leukemia cell differentiation; however, it inhibits ES cells differentiation and present at day 4 dpc during the mouse development (Hilton, 1992). The *myb* gene family coding for transcription factors have previously been shown to be essential for myeloid cell differentiation, in particular, c-*myb* (section 1.2.4) was expressed at high levels in immature hematopoietic cells and decreased in expression during terminal differentiation. In embryo, the expression of c-*myb* was very low before 10.5 dpc but detected from 10.5 dpc onwards and at a similar level throughout all stages of embryonic development. On close examination, c-*myb* is strongly expressed in the developing liver and thymus (but not yolk sac) at 10 dpc (hematopoietic organs), and non-hematopoietic tissues like nasal region, respiratory tract and the developing eyes (Sitzmann *et al.*, 1995). Another myeloid specific DNA binding protein (PEBP2αB/CBFα) involved in myeloid leukemia and myelopoiesis (Okuda *et al.*, 1996) was also found to be important in embryogenesis. Its Drosophila homolog *runt* (a pair-rule gene) is involved in defining compartments of the embryo. In mouse embryo, CBFα expression was detected from 9.5 dpc and the highest levels of expression were found in the neural tube, sensory ganglia, specialized sensory epithelial structures, all chondrogenic centres, genital system and thymus from 10.5

dpc (Simeone *et al.*, 1995). These examples clearly demonstrated that genes that first appeared to be restricted to myeloid tissues or hematopoietic tissue, may also be involved in the development of other tissues in embryos.

Another group of genes, *hox,* are involved in myeloid cell differentiation (Lill *et al.*, 1995; Shen *et al.*, 1992; Wu *et al.*, 1992); they do not seem to contribute to cell differentiation and/or proliferation events in embryo. *hox* are arranged in 4 clusters. Their order of alignment along the 5' to 3' end define their expression on the anterior-posterior axis which are responsible for determining segment identity during embryogeneisis.

Briefly, the isolated gene fragments obtained as described in Chapter 3 can be characterized preliminary in the following ways. First, by sequencing and homology search, we can see if these fragments belong to a known gene or not. Following that, the expression pattern of these genes in midazolam-induced JCS cells can be confirmed by a fast and sensitive method, RT-PCR.

Further, studying the expression profile of these genes in biochanin A-treated cells may be rewarding. As discussed in section 1.2.4, certain genes (e.g. *egr-1* and HOX B7 gene) may have different effects on cells committed to different lineages (Lill *et al.*, 1995; Nguyen *et al.*, 1993). By comparing the expression pattern of these genes in midazolam-induced (committed to both monocytic and granulocytic differentiation) and biochanin A-induced (committed to monocytic differentiation) JCS cells, the differential effect of these genes on lineage commitment may be revealed.

Lastly, to increase our understanding of the isolated genes (especially for those putative novel genes), their expression pattern during embryonic development are proposed to be studied.

4.2 Materials

(See also earlier chapters for materials which have already been mentioned)

4.2.1 Selected recombinant plasmids

17 isolated recombinant plasmids were selected for further studies in this Chapter, namely, 44/3.1, 147/1.4, 147/1.5, 147/1.6, 172/2.8, 172/3.1, 172/3.2, 172/3.8, 172/4.1, 172/4.6, 172/5.2, 172/5.6, 172/5.8, 228/5.1, 228/5.2, 228/5.3 and 228/5.6 (Table 3.4)

4.2.2 Total RNAs

1. Total RNAs from biochanin-A induced JCS cells at time point 0, 1, 5, 18 and 46 hours kindly prepared by Miss Pandora, M.C. Yip in our laboratory.
2. Total RNAs prepared from midazolam-induced JCS cells.
   Total RNAs from embryo at different developmental stages generously provided by Miss Jessie, P. K. Lee in our laboratory. The embryos were obtained from pregnant ICR mice in the Animal House of the Chinese University of Hong Kong. Decidum (from 7.5 dpc embryos) and placenta (from 9.5, 11.5, 15.5 and 17.5 dpc embryo) were removed.

4.2.3 Chemicals

1. Ammonium persulfate	Pharmacia biotech plus one 17-1311-01
2. Boric acid	Pharmacia 80-1128-86
3. EDTA, disodium salt	Pharmacia 80-1128-85
4. Primer oligo (see also Table 4.5)	Integrated DNA Technologies
5. ReadyMix Gel, ALF grade	Pharmacia 17-1035-01
6. Sodium acetate	Sigma S8750

7. Tris	Pharmacia 80-1128-82

4.2.4 Enzymes and nucleic acids

1. Bam HI, 10X buffer K	Amersham E1010YH
2. Pst I, 10X buffer H	Amersham E1073YH

4.2.5 Kits (Appendix A3)

1. AutoRead sequencing kit	Pharmacia XY-056-00-02

4.2.6 Solutions

1. Sodium acetate, 3M, pH 4.8	Aqueous sodium acetate with pH adjusted by glacial acetic acid
2. TBE, 10X	1 M Tris, 0.85 M Boric acid, 10 mM EDTA

4.3 Methods

4.3.1 Preparation of selected recombinant plasmid DNA

Plasmid DNA of each isolated clone was prepared from 50 ml of bacterial culture using the Qiagen column as described in section 3.3.5(i). Each Qiagen column was used to purify the plasmid DNA from 25 ml of bacterial culture (section 3.3.5(i)) and regenerated by 5 ml of additional QF solution. The regenerated column was immediately used to purify plasmid DNA from another 25 ml of bacterial culture. The used Qiagen columns were washed with 5 ml of QF solution, submerged using excess volume of 70 % ethanol and stored at 4 °C. A collection of the treated columns were rinsed with distilled water, autoclaved, dried at 37 °C and kept at room temperature for future use.

1 μg of each isolated plasmid was digested by Bam H1 alone or by Bam HI + Pst I for 1 hour at 37 °C.

4.3.2 Sequencing

(i) Preparation of gel cassette and sequencing gel

Thermostatic plate, notched glass plate, spacers and comb (A.L.F.™ DNA sequencer) were thoroughly cleaned with non-fluorescent detergent, rinsed with running tap water followed by Ultrapure water and wiped with lint-free Kimwipe as suggested by the manufacturer. Then, the plates were rinsed with 70 % commercial grade ethanol and polished dry with Kimwipe. After that, they were rinsed again with ultrapure water and polished dry with Kimwipe. This ethanol-water cycle was repeated for three times. The items were then carefully examined under reflected light to ensure no gel residues and dust remained. The light coupler was cleaned with ultrapure water and dried with Kimwipe and should be free of crystals, cracks and

marks on the polished ends. The gel cassette was then assembled with thermoplate, notched plates, spacer and light coupler in place fastened by four gel clamps on a horizontal support.

A bottle of ReadyMix gel removed from 4 °C was equilibrated to room temperature and added with 450 µl of 10 % (w/v) freshly prepared ammonium persulfate. The solution was then mixed by gentle rocking the bottle in a horizontal manner for 30 seconds without introducing bubbles. The solution was subsequently applied into the gel cassette from the top side to make a 0.5 mm thick gel. The cassette was slightly tapped just behind the gel front for an even spread of gel solution; any bubbles introduced were removed by a thin plastic strip. The comb was inserted in position and the gel was left for polymerization (45 minutes). According to the instructor's manual, the upper reservoir was attached to the electrophoresis unit. This unit was in turn rested on the lower reservoir connecting to the anode. The gel assembly was then connected to the cathode and the inlet/outlet of the thermoplate. Both reservoirs were filled with 0.6 X TBE buffer. The laser beam was tuned to maximum intensity passing through the centre of each detector by visual inspection. The electrophoresis conditions were set inside the ALF manager v 2.6 (Pharmacia Biotech) programme. The conditions were voltage = 1500 V, current = 38 mA, power = 34 W, temperature = 40 °C and laser power = 3 mW. The sampling interval was 2 seconds and the running time was 6 hours. The assembly was then ready for sample loading.

(ii) Sequencing reaction

The plasmids with the subcloned fragments were first diluted to a concentration of 5-10 µg / 32 µl as suggested in the instructor manual of the AutoRead sequencing kit (Pharmacia) (see also Appendix A3). Two sets of solution were prepared for the M13 universal and the reverse primer reaction. The template solution was then added with 8 µl of 2M NaOH. The mixture was vortexed, centrifuged to bring down droplets on the cap and incubated at room temperature for 10 minutes. 7 µl of 3 M sodium acetate, pH 4.8 plus 4 µl of ultrapure H_2O were

added to neutralize the solution. 120 μl of 100% ethanol was then added, mixed and incubated on ice for 15 minutes. The reaction solution was centrifuged at 14 000 rpm for 15 minutes in a bench-top microcentrifuge (Eppendorf). With the supernatant discarded, the pellets were rinsed with 70% ethanol and centrifuged at 14 000 rpm for further 15 minutes. The supernatant was removed and the pellets were dried in vacuum (Savant). The dried pellets were resuspended in 10 μl of H_2O and mixed with 2 μl of fluorescent primer (either the M13 universal or the reverse primer) and 2 μl of annealing buffer to make the annealing reaction solution. The mixture was vortexed, centrifuged for a while and pre-heated at 65 °C for 5 minutes. It was then immediately incubated for 10 minutes at 37 °C and left at room temperature for 10 minutes. Further 1 μl of extension buffer, 3 μl of DMSO and 8U/2μl diluted T7 DNA polymerase were added to the annealing reaction solution. For each sequencing reaction, 2.5 μl of each sequencing mix (A, T, C, G) was pipetted into separate eppendorf tubes and incubated on ice for 5 minutes. The tubes were then transferred to a 37 °C heat block (Thermolyne) for at least 1 minute. 4.5 μl of the annealing reaction solution with T7 DNA polymerase added was dispensed to each sequencing mix and incubated at 37 °C for 5 minutes. 5 μl of stop solution with loading dyes was subsequently added to each tube and kept on ice until loading. Just before the reaction solutions were loaded into the gel, the reaction mix were heated to 90 °C for 3 minutes to detach the chain-terminated molecules and snap-freezed onto the ice. The well of the gel was washed twice with 0.6 X TBE buffer and 8 μl of each mix was loaded into the gel.

4.3.3 Data analysis and assessment by ALF manager and DNAsis

Every four lanes of the A, C, G, T reactions were grouped and named as a clone. The fluorescent signal of the four lanes in each clone were detected by the photodetectors during the electrophoresis and converted into serial digital data. The data were fed into the computer and saved in a file (ALF file). The data was also processed and the base sequences were determined according to the intensity peaks of the four bases relative to each other on the time scale. The sequence of the clone was

then exported in ASC II format and further analyzed using the software DNAsis for Window (Hitachi).

The forward sequence of a clone extended from the M13 universal primer and the reverse sequence extended from the M13 reverse primer were connected together through the function Contig Manager in the DNAsis program with a minimum overlap of 30 base pair and a minimum matching of 90 % within the overlapping region. A consensus sequence with the overlapping sequences merged was generated. In the case of unmatched base in the overlapping region, an ambiguity code would be reported in that position. The consensus sequence was then saved in ASC II format. The vector sequence, the region between the sequencing primer and the cloning site, was removed manually by visual examination from the file. The resulting sequence was then ready for primer design and matching in the Genbank. If the forward and reverse sequences could not be connected in the middle, they were artificially joined in the same orientation with a middle gap in between. A pair of primers were designed across the gap for further investigation.

4.3.4 Sequence search by BLASTN program

The consensus sequence in ASC II format (section 4.3.4) was kept in files for BLAST E-mail search requested at the address blast@ncbi.nlm.nih.gov. Only program 'blastn' matching the input DNA sequence with the DNA sequence database was used in this investigation. The subject line was left blank. The search command begins with the mandatory parameter 'PROGRAM' followed by the value blastn. The next line was the mandatory search parameter 'DATALIB' and the value was input as 'nr' standing for non-redundant database. The third line is an optional parameter 'EXPECT' and our value was set at 0.75. The fourth line was the mandatory 'BEGIN' directive indicating the beginning of the query sequence. This was followed by a filename and the query sequence was enclosed with the E-mail message. The following demonstrates a typical request mail, the letters in bold being automatically created by the E-mail program.

From : yyszeto@cuhk.edu.hk
Date : 29 June 1996 12:00:00
To : blast@ncbi.nlm.nih.gov
Subject :

Message :

PROGRAM blastn
DATALIB nr
EXPECT 0.75
BEGIN
> filename.seq
CTAGCTGGGACGGCTACGTACCTTAAATCATCACCAGTAGAGTTCCCCCAT
ACCCCTATCTAGACTGCTTGACGGTCTAACTGCGTACATTGACTCGAGACC
CCGATAGCTTAGCGGTA

4.3.5 Primer design by Oligo™ ver. 3.4

The same sequence file (section 4.3.4) in ASC II format was retrieved in the software OLIGO™ (National BioSciences). Primers were selected internal to the arbitrary primers used to generated the fragment concerned. Several parameters were checked in the software including false priming, internal stability, primer dimer formation and self-complementarity; the melting temperature (Tm) of each primer was kept between 62-66 °C calculated from the formula 2 °C(A+T) + 4 °C(G+C). The difference of Tm between each pair of primers was kept at a maximum of 2 °C.

4.3.6 Differential expression confirmed by RT-PCR

After specific primer pairs were designed, RT-PCR were performed as described in section 2.3.2 to compare the expression level of each gene represented by the isolated gene fragments in JCS cells induced by midazolam at various time points (0, 1, 5, 18 and 48 hours). Two to three different cycle numbers ranging from 20-35 were done for each gene fragment depending on the expression level of individual gene. The expression level of these gene fragments were also studied in other samples : JCS cells treated with biochanin A for 0, 1, 5, 18 and 46 hours and embryos at different developmental stages (7.5, 9.5, 11.5, 15.5, 17.5 dpc).

4.4 Results

4.4.1 Analysis of selected recombinant plasmid DNA

Preparation of recombinant plasmid DNA

DNA of each recombinant plasmid was prepared from 50 ml of bacterial culture as described in section 4.3.1. The yield of each preparation was also estimated according to 1 unit A_{260} = 50 μg/ml double-stranded DNA. The ratio A_{260} to A_{280} among the different preparations varied from 1.4 to 1.6 and the yield (μg) per 50 ml of bacterial culture also ranged from 13.2 to 288 as shown in Table 4.2.

Table 4.2 : **Spectrophotometric analysis of recombinant plasmid preparation.**

Fragment no.	A_{260}/A_{280}	yield (μg)/ 50 ml culture
44/3.1	1.367	234
147/1.4	1.381	110
147/1.5	1.592	13.2
147/1.6	1.623	14
172/2.8	1.378	86
172/3.1	1.424	104
172/3.2	1.441	105
172/3.8	1.423	204
172/4.1	1.382	104
172/4.6	1.455	122
172/5.2	1.326	170
172/5.6	1.399	288
172/5.8	1.376	194
228/5.1	1.341	150
228/5.2	1.632	168
228/5.3	1.420	116
228/5.6	1.370	112

Plasmids harboring subcloned fragments as indicated were isolated by the Qiagen plasmid midi kit and the column was used 2 times consecutively in one preparation for 50 ml culture (section 4.3.1). Absorbance at 260 nm to 280 nm (A_{260} / A_{280}) and the yield / 50 ml culture were also calculated (see also text).

Restriction digestion of the subcloned fragments from the plasmid vector

The size of insert in each recombinant plasmid was analyzed by Bam H1 and Pst I restriction enzyme digestions. Bam HI and Pst I sites are two unique restriction sites closest to the chosen cloning site Sma I; thus the incised fragments gave an approximation of the size of the subcloned fragments. Two incised fragments were found in plasmids containing fragment 147/1.4, 172/3.8 and 172/5.6. The estimated size of the incised fragments were summarized in Table 4.3. The mobility of the linear recombinant plasmids (Figure 4.2, lane 2) relative to the plasmids with the inserts cut were shown (Figure 4.2, larger band, lane 1). Notable decreases in mobility at lane 2 were found in plasmid harboring fragment 44/3.1, 172/2.8 and 172/3.8. The subcloned fragments were relatively large in size (>500 bp). Two small incised bands were also found in lane 2 of fragment 172/5.6 containing plasmid where only Bam HI was added for digestion. Lane 3 (Figure 4.2) showed the undigested plasmid having negligible contamination of genomic DNA and undetectable level of RNA. The preparations also contained rather intact plasmids where linear form or nicked form was not detectable.

Table 4.3. **Sizes of the subcloned fragments estimated by restriction enzyme digestion or determined by sequencing.**

Gene fragments	Sizes of the fragment* (estimated by restriction digestion, BamHI + Pst I)	Sizes of the fragments (bp) (determined by sequencing)
44/3.1	870	>690 (690 with gap in between)
147/1.4	239+208 = 447	393
147/1.5	479	>315 (315 with gap in between)
147/1.6	389	311
172/2.8	512	515
172/3.1	398	393
172/3.2	275+186 = 461	413
172/3.8	749	840
172/4.1	213	197
172/4.6	371	397
172/5.2	323	324
172/5.6	372+245 = 617	659
172/5.8	339	397
228/5.1	316	>263 (263 with gap in between)
228/5.2	417	340
228/5.3	389	301
228/5.6	389	300

*1 μg of each recombinant plasmid was digested by Bam HI and Pst I for 1 hour at 37 °C. The digested fragment was loaded into a 1 % TAE agarose gel. The fragment size was estimated from the migrating distance by the standard curve **Log_{10} (nucleotide pair) = 4.5-0.59 (distance migrated in cm)** constructed by the marker standard loaded in parallel. The actual fragment size determined by sequencing is illustrated and compared.

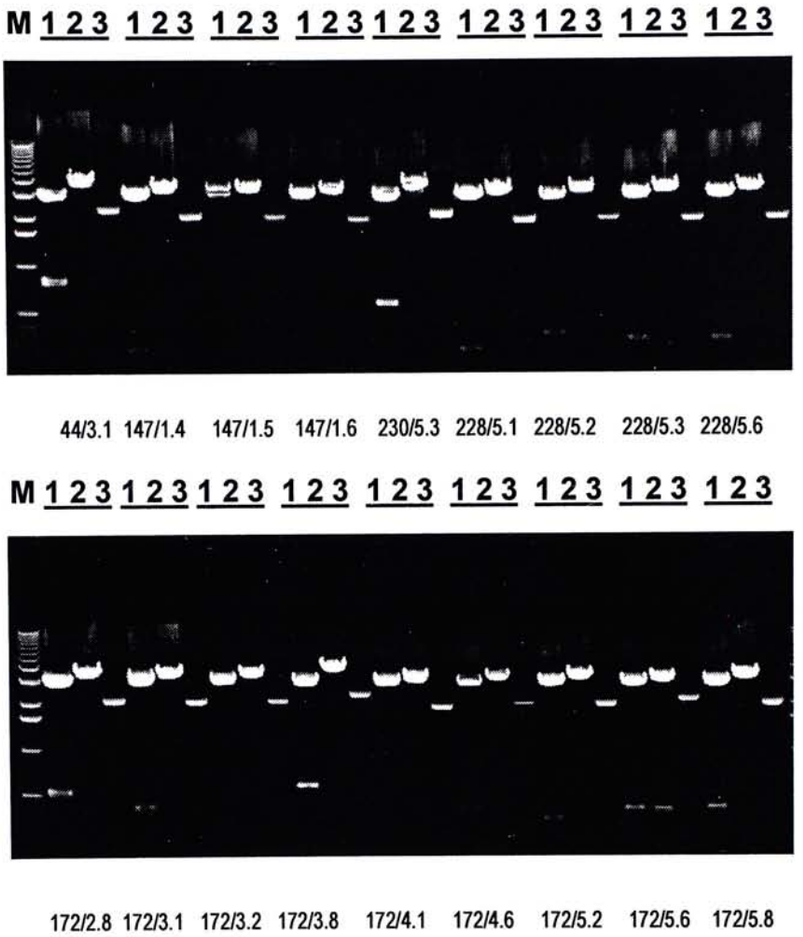


Figure 4.2. **Digestion of subcloned fragments inserted at Sma I from the plasmid vector pBluescript SK-.** 1 μg of recombinant plasmid digested by Bam HI + Pst I (lane 1), Bam H1 alone (lane 2) or 0.3 μg plasmid DNA without digestion (lane 3) were electrophoresed on a 1 % TAE agarose gel. Names of the fragment were designated as follows : arbitrary primer number/fragment number assigned in RAP-PCR . clone number assigned according to the different sizes of inserts. M=1Kb ladder.

4.4.2 Sequencing results

Automated sequencing by ALF system was done for the following 17 fragments. Double-stranded sequencing following the protocol in the AutoRead sequencing kit (see also section 4.3.2 (ii)) was performed. Recombinant plasmids of pBluescript SK- inserted with each of these fragments were denatured and annealed with either a M13 fluorescent universal or reverse primer. 4 separate reactions for each nucleotide A, C, G, T were performed and loaded separately on different lanes on the gel. The fluorescent signals from each lane were detected, analyzed, saved in an ALF file and presented as curves (electropherograms) (see also section 4.3.3) (Figure 4.3). The system could read 270-580 bp without ambiguities and an average of about 400 bp under the methods described and the fragment used in this investigation. The sequencing result in ASC II format of the same fragment initiated by the universal primer and the reverse primer were combined together as described in section 4.3.3. Sequences that did not meet in the middle were artificially joined in the same orientation. In the following, `********` indicates the sequence of the arbitrarily chosen primer used to generate the sequence. This short sequence occurred at both ends. `---GAP---` indicates the undetermined sequences of unknown size stuffing in between. `-------->` indicates the upper primer designed for further experiments (RT-PCR) while `<--------` indicates the lower primer (see also Table 2.5). A, C, T, G represents the four dideoxynucleotides and ambiguity codes were explained in Appendix A1. The length of these subcloned fragments were summarized in Table 4.3.

Figure 4.3.(opposite page) **A typical output of automated sequencing by ALF system.** The fluorescent signals from each of the four lanes, A, C, G, T were detected, analyzed and presented as electrophoregram. Green line : A; Blue line : C; Black line, G; Red line, T

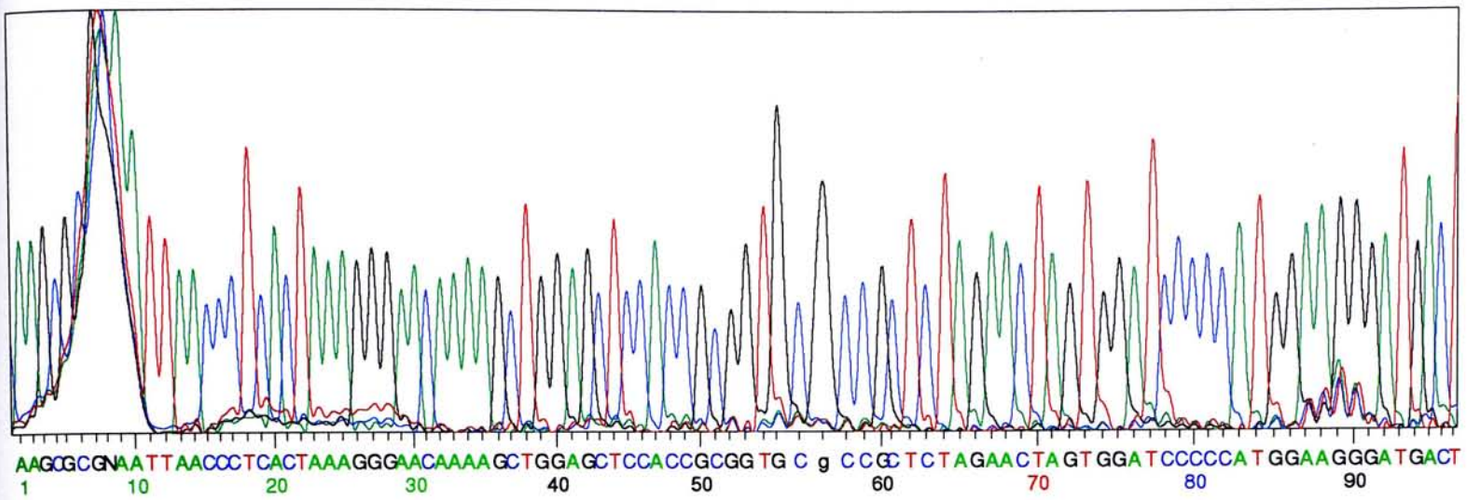
File: A:\IRENE4A.ALF

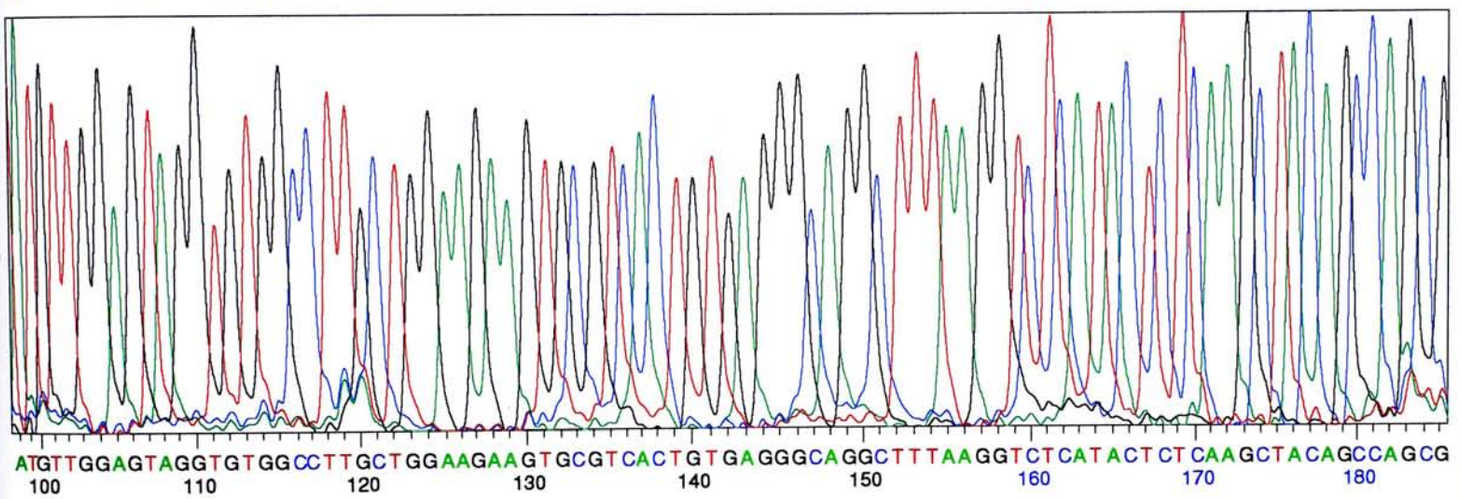
Clone: 4, (Sc) (P)

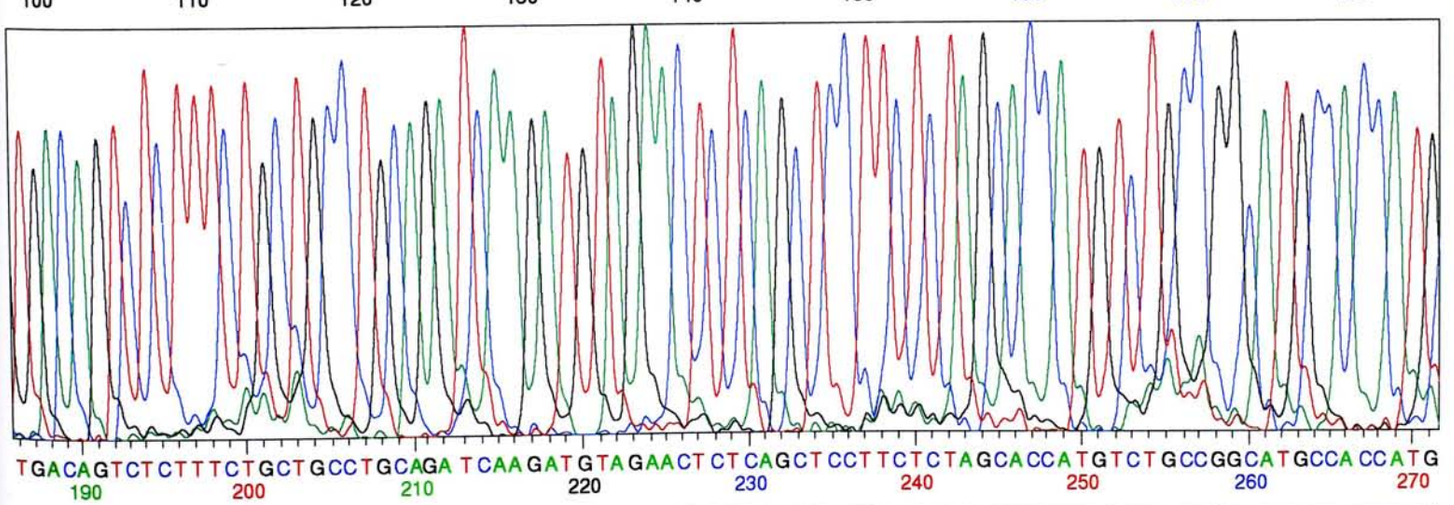
Run:

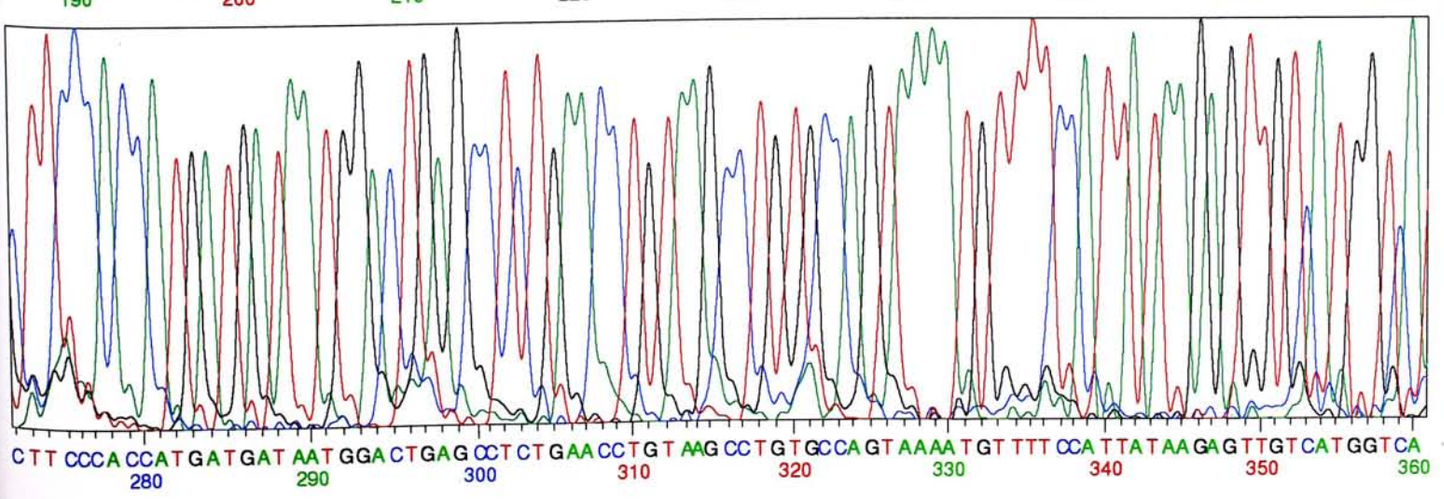
Time: 0:57 - 4:08 [h:m]

Date: 03-12-96









Comment:

Operator:

Fragment 44/3.1

Length of the fragment : estimated to be 870 bp (Table 4.3)

Arbitrary primer : 5′ATA CAC GAT TTT CCA TCA CAA ACT 3′

```
        10          20          30          40          50          60
************************                                       ---
TACACGATTT TCCATCACAA ACGGTCTCAG TCCCTGCACA TGTCTGTGAA AATTATTTAA
ATGTGCTAAA AGGTAGTGTT TGCCAGAGTC AGGGACGTGT ACAGACACTT TTAATAAATT

        70          80          90         100         110         120
-------------------->
ATGAGGTAAC TGATGTGGGA CAACCTACTA TAAGTATGGG CTATATGTTA CAATAGGTGA
TACTCCATTG ACTACACCCT GTTGGATGAT ATTCATACCC GATATACAAT GTTATCCACT

       130         140         150         160         170         180
TTATACTGCT CAAATCACCC CACAGTTGTT AGTTGTTGGC ATATATTCCT TGCTGCCCTC
AATATGACGA GTTTAGTGGG GTGTCAACAA TCAACAACCG TATATAAGGA ACGACGGGAG

       190         200         210         220         230         240
ATCAATGTTC TGGAAATGAA AGACACACAC CTGACATTTT GATCTTAGCC ATTCTAACTG
TAGTTACAAG ACCTTTACTT TCTGTGTGTG GACTGTAAAA CTAGAATCGG TAAGATTGAC

       250         260         270         280         290         300
GTAAAAGATA GAATCTCAAG GTCATTTTGA TTTGCATTTC TCTGATCACT AAGAACATTT
CATTTTCTAT CTTAGAGTTC CAGTAAAACT AAACGTAAAG AGACTAGTGA TTCTTGTAAA
                                  <--------------------------

       310         320         330         340         350         360
                                                                     -
CTTTAGGTGC TTCTCAGCCM aTBCGAGATY C--------GAP--------- GTGGAAADVC
GAAATCCACG AAGAGTCGGK tAVGCTCTAR G--------GAP--------- CACCTTTHBG

       370         380         390         400         410         420
-------------------->
CCCCATGAGA TCTAGCTGTA AGGCATTTTC TCAATTAGTG ATCAAGGGGG AAAGGCCCCT
GGGGTACTCT AGATCGACAT TCCGTAAAAG AGTTAATCAC TAGTTCCCCC TTTCCGGGGA

       430         440         450         460         470         480
TGTGGGKGGG ACCATCTCTG GACTGGCAGT CTTGGGTTCT ATAAGAGAGC AGGCTGAGCA
ACACCCMCCC TGGTAGAGAC CTGACCGTCA GAACCCAAGA TATTCTCTCG TCCGACTCGT

       490         500         510         520         530         540
AGCCAGGGGA GGCAAGATAG TAAGGAACAT CCCTTCATGG CCTCTGCATC AGCTCCTGCT
TCGGTCCCCT CCGTTCTATC ATTCCTTGTA GGGAAGTACC GGAGACGTAG TCGAGGACGA

       550         560         570         580         590         600
TCCTGACCTG CTTGAGTTCC AGGCCTGACT TCCTTTGGTG ATGAACAGCA GCATGGAAGT
AGGACTGGAC GAACTCAAGG TCCGGACTGA AGGAAACCAC TACTTGTCGT CGTACCTTCA

       610         620         630         640         650         660
GTAAGCCAAA TAAACCCTTT CCTCCCCAAC TCGCTTCTTG GTCATGATGT TTGTGCAGGA
CATTCGGTTT ATTTGGGAAA GGAGGGGTTG AGCGAAGAAC CAGTACTACA AACACGTCCT
                                  <----------------------

       670         680         690         700         710         720
ATAGAGACCT TGACTAAGAC AGGTCGTTTG TGATGGAAAA TCGTGTAT
TATCTCTGGA ACTGATTCTG TCCAGCAAAC ACTACCTTTT AGCACATA
                      **************************
```

Fragment 147/1.4

Length of the fragment =393 bp

Arbitrary primer used : 5′ATG ATG CTC TTT AGG CTT TCC AG 3′

```
        10          20          30          40          50          60
************************
ATGATGCTCT TAGGCTTTCC AGAAGAGGGT GTCAGATCTC ATTACGGATG GTTGTAAGCC
TACTACGAGA ATCCGAAAGG TCTTCTCCCA CAGTCTAGAG TAATGCCTAC CAACATTCGG

        70          80          90         100         110         120
  ---------------------->
ACCATGTGGT TGCTGGGATT TGAACTCAGG ACCTTTGGAA GAGCAGTCGG TGCTCTTAAC
TGGTACACCA ACGACCCTAA ACTTGAGTCC TGGAAACCTT CTCGTCAGCC ACGAGAATTG

       130         140         150         160         170         180
CATTGAGCCG TCTCTCCAGC CCCTCAACAC YGTAAACYTA CYTAAGCATG AACYAAATTT
GTAACTCGGC AGAGAGGTCG GGGAGTTGTG RCATTTGRAT GRATTCGTAC TTGRTTTAAA

       190         200         210         220         230         240
AAAAATTCTT TGACATTTAC YTAWTCATAG ATKGTATGTG GTAGTKGAGG TKGGGGATSC
TTTTTAAGAA ACTGTAAATG RATWAGTATC TAMCATACAC CATCAMCTCC AMCCCCTASG

       250         260         270         280         290         300
TGCAGAGCCT TGTGTGGAGG TCAGAGGGGA GGTAGAGGGG GTCGGTTCTC AGACATGTGA
ACGTCTCGGA ACACACCTCC AGTCTCCCCT CCATCTCCCC CAGCCAAGAG TCTGTACACT

       310         320         330         340         350         360
CTGTGTGACA GACTGCAAGC AGGCAGAGAA CATCTCTCTT TGTCTTCTTG TGACATGCTG
GACACACTGT CTGACGTTCG TCCGTCTCTT GTAGAGAGAA ACAGAAGAAC ACTGTACGAC
                                  <-----------------------

       370         380         390         400         410         420
TCATTGTCAG CTGGAAAGCC TAAAGAGCAT CAT
AGTAACAGTC GACCTTTCGG ATTTCTCGTA GTA
          *************************
```

Fragment 147/1.5

Lenght of the fragment = estimated to be 479 bp (Table 4.3)

Arbitrary primer used : 5′ATG ATG CTC TTT AGG CTT TCC AG 3′

```
        10          20          30          40          50          60
********************** ---------------------->
TGATGCTCTT TAGGCTTTCC AGACCCCCAA AATCAGAGTA GACATATTAG AAAGTAAAAC
ACTACGAGAA ATCCGAAAGG TCTGGGGGTT TTAGTCTCAT CTGTATAATC TTTCATTTTG

        70          80          90         100         110         120
CCACAGTGCA CTCTCCTGCC TGTGGCCAGA CTTGGACTTC TCTCCTCTAA AGAAAAGAGG
GGTGTCACGT GAGAGGACGG ACACCGGTCT GAACCTGAAG AGAGGAGATT TCTTTTCTCC

       130         140         150         160         170         180
TCGACTWAAG GGAAGAAGCC TTyCARGCYT ---------GAP--------- CTAACCTTGG
AGCTGAWTTC CCTTCTTCGG AArGTYCGRA ---------GAP--------- GATTGGAACC

       190         200         210         220         230         240
TTTGGRAARG GGGTTTTTKG SCTTCCCAGG TTACAGTCCA TCATCATGGG ATATCTGTAT
AAACCYTTYC CCCAAAAAMC SGAAGGGTCC AATGTCAGGT AGTAGTACCC TATAGACATA

       250         260         270         280         290         300
AAACTGCCAA CACGCCCACT CCATGGTGTG GGAAGGTTTT ATTGTAGAGG AGAGGGAGAG
TTTGACGGTT GTGCGGGTGA GGTACCACAC CCTTCCAAAA TAACATCTCC TCTCCCTCTC
                                                       <--------

       310         320         330         340         350         360
CAGCCAGAAG CATCTGGAAA GCCAAAGAGC ATCAT..... .......... ..........
GTCGGTCTTC GTAGACCTTT CGGTTTCTCG TAGTA..... .......... ..........
------------  ************************
```

Fragment 147/1.6

Length of the fragment = 311 bp

Arbitrary primer used : 5′ATG ATG CTC TTT AGG CTT TCC AG 3′

```
        10          20          30          40          50          60
                                ---------------------->
**************************
ATGATGCTCT TTAGGCTTTC CAGAAGCACC ACTCTCAGGG GAATCCAAGG CCACTGCTTC
TACTACGAGA AATCCGAAAG GTCTTCGTGG TGAGAGTCCC CTTAGGTTCC GGTGACGAAG

        70          80          90          100         110         120
TCTGGCCCTK ATTCACTGTT TGATAGTAAA CTGGACTTAC CTKAAATTAG TCCCAGAGGT
AGACCGGGAM TAAGTGACAA ACTATCATTT GACCTGAATG GAMTTTAATC AGGGTCTCCA

       130         140         150         160         170         180
GGGTCCTTCT GTCACTGAGT TCATATGCTT KGCCCCTGCC AGTACCACTG GGGAAACAGT
CCCAGGAAGA CAGTGACTCA AGTATACGAA MCGGGGACGG TCATGGTGAC CCCTTTGTCA

       190         200         210         220         230         240
CACTAATGCT ATGGTWTCTA CCCATAGAAC TACTGGTTGA CTCTGCATGG GGTAGTACTG
GTGATTACGA TACCAWAGAT GGGTATCTTG ATGACCAACT GAGACGTACC CCATCATGAC
                                            <----------------

       250         260         270         280         290         300
TTTGGAGTMA CAGCTCTTGA GCTGGCATTA GACAGACTCT GTGTGGTCCT GGAAAGCCTA
AAACCTCAKT GTCGAGAACT CGACCGTAAT CTGTCTGAGA CACACCAGGA CCTTTCGGAT
------                                      **************

       310         320         330         340         350         360
AAGAGCATCA T
TTCTCGTAGT A
************
```

Fragment 172/2.8

Length of the fragment = 515 bp

Arbitrary primer used : 5′ATG GAA GGG ATG ACT ATG TTG GA 3′

```
        10          20          30          40          50          60
************************
ATGGAAGGGA TGACTATGTT GGAGTAGGTG TATCACTGTG GATGTGGGCT TTACGATCCT
TACCTTCCCT ACTGATACAA CCTCATCCAC ATAGTGACAC CTACACCCGA AATGCTAGGA

        70          80          90          100         110         120
CATCCTAGCT TCCCAGAAGC CAGTATTCCA CTAGCAGCCT TCAGATGAAG ATGTAGAACT
GTAGGATCGA AGGGTCTTCG GTCATAAGGT GATCGTCGGA AGTCTACTTC TACATCTTGA

       130         140         150         160         170         180
CTCAGCTCCT TCTCTAGCAC CATGTCTGCC GGCATGCCAC CATGCTTCCC ACCATGATGA
GAGTCGAGGA AGAGATCGTG GTACAGACGG CCGTACGGTG GTACGAAGGG TGGTACTACT

       190         200         210         220         230         240
TAATGGACTG AACCTCTGAA CCTGTAAGCC TGTVCCAGTA AAATGTTTTC CATTATAAGA
ATTACCTGAC TTGGAGACTT GGACATTCGG ACABGGTCAT TTTACAAAAG GTAATATTCT

       250         260         270         280         290         300
GTTGTCATGG TCATGGTGTC TCTTCAGAGC AGTAAAACCC TCACTAAGAG AGTTGTGTTG
CAACAGTACC AGTACCACAG AGAAGTCTCG TCATTTTGGG AGTGATTCTC TCAACACAAC

       310         320         330         340         350         360
CATGGATCAG GACTGTVGCT CACTTAGGAG GNCVNGAGGA AGATGTCACC CTGGTAACTC
GTACCTAGTC CTGACABCGA GTGAATCCTC CNGBNCTCCT TCTACAGTGG GACCATTGAG

       370         380         390         400         410         420
CAGGTTCCAA CATAGTCATC CCTTCCATAA CTCTGCTAAT GAAGTGGATC TTCTGGGYCC
GTCCAAGGTT GTATCAGTAG GGAAGGTATT GAGACGATTA CTTCACCTAG AAGACCCRGG
     ************************
       430         440         450         460         470         480
AAACCAGAAT GGTTCTGAGG GCTTAGCCCR AGCTGACSCR AGVCSAATGG TGCCAAGCCT
TTTGGTCTTA CCAAGACTCC CGAATCGGGY TCGACTGSGY TCBGSTTACC ACGGTTCGGA

       490         500         510         520         530         540
GTGGAGGATT TCTCCAACAT AGTCATCNNT DTVAT
CACCTCCTAA AGAGGTTGTA TCAGTAGNNA HGGTA
            ************************
```

Fragment 172/3.1

Length of the fragment = 393 bp

Arbitrary primer used : 5′ATG GAA GGG ATG ACT ATG TTG GA 3′

```
        10          20          30          40          50          60
**************************
ATGGAAGGGA TGACTATGTT GGACTAGGTG TGCCACTGTG GGAGTGGGCT TTGAGACCCT
TACCTTCCCT ACTGATACAA CCTGATCCAC ACGGTGACAC CCTCACCCGA AACTCTGGGA

        70          80          90         100         110         120
CCTTCTAGCC GGGTGGGACG AAGCCCAGTC TTCTTCTGGG TTCCCTTGAA TAAGACATGG
GGAAGATCGG CCCACCCTGC TTCGGGTCAG AAGAAGACCC AAGGGAACTT ATTCTGTACC

       130         140         150         160         170         180
AACTCTCAGC TCCTTCTCTA GCGCCATGTC YTGCCGGCAT GCCACCATGY TTCCCACCAT
TTGAGAGTCG AGGAAGAGAT CGCGGTACAG RACGGCCGTA CGGTGGTACR AAGGGTGGTA

       190         200         210         220         230         240
GATGATAATG GACTGAACCT CTGAACCTGT AAGCCTGTGC CAGKAAAAKG TTTTCCATTA
CTACTATTAC CTGACTTGGA GACTTGGACA TTCGGACACG GTCMTTTTMC AAAAGGTAAT

       250         260         270         280         290         300
TAAGAGTTKT CATGGTCATG GTGTCTCTTC AGAGCAGTAA AACCCTCACT AAGAGAGTTG
ATTCTCAAMA GTACCAGTAC CACAGAGAAG TCTCGTCATT TTGGGAGTGA TTCTCTCAAC

       310         320         330         340         350         360
TGTTGCATGG ATCAGGACTG TGGCTCACTT AGGAGGTCAT GAGGAAGATG TCACCCTGGT
ACAACGTACC TAGTCCTGAC ACCGAGTGAA TCCTCCAGTA CTCCTTCTAC AGTGGGACCA

       370         380         390         400         410         420
AACTCCAGGT TCCAACATAG TCATCCCTTC CAT
TTGAGGTCCA AGGTTGTATC AGTAGGGAAG GTA
          **************************
```

Fragment 172/3.2

Length of the fragment = 413 bp

Arbitrary primer used : 5′ATG GAA GGG ATG ACT ATG TTG GA 3′

```
        10          20          30          40          50          60
**************************              ---------------------->
ATGGAAGGGA TGACTATGTT GGAGTAGGTG TGGCCTTGCT GGAAGAAGTG CGTCACTGTG
TACCTTCCCT ACTGATACAA CCTCATCCAC ACCGGAACGA CCTTCTTCAC GCAGTGACAC

        70          80          90         100         110         120
AGGGCAGGCT TTAAGGTCTC ATACTCTCAA GCTACAGCCA GCGTGACAGT CTCTTTCTGC
TCCCGTCCGA AATTCCAGAG TATGAGAGTT CGATGTCGGT CGCACTGTCA GAGAAAGACG

       130         140         150         160         170         180
TGCCTGCAGA TCAAGATGTA GAACTCTCAG CTCCTTCTCT AGCACCATGT CTGCCGGNAT
ACGGACGTCT AGTTCTACAT CTTGAGAGTC GAGGAAGAGA TCGTGGTACA GACGGCCNTA

       190         200         210         220         230         240
GCCACCATGC TTCCCACCAT GATGATAATG GACTGAGCCT CTGAACCTGT AAGCCTGTGC
CGGTGGTACG AAGGGTGGTA CTACTATTAC CTGACTCGGA GACTTGGACA TTCGGACACG

       250         260         270         280         290         300
CAGTAAAATG TTTTCCATTA TAAGAGTTGT CATGGTCATG GTGTCTCTTC AGAGCAGTAA
GTCATTTTAC AAAAGGTAAT ATTCTCAACA GTACCAGTAC CACAGAGAAG TCTCGTCATT

       310         320         330         340         350         360
AACCCTCACT AAGAGAGTTG TGTTGCATGG ATCAGGACTG TGGCTCACTT AGGAGGTCAT
TTGGGAGTGA TTCTCTCAAC ACAACGTACC TAGTCCTGAC ACCGAGTGAA TCCTCCAGTA
                <-----------------------
       370         380         390         400         410         420
GAGGAAGATG TCACCCTGGT AACTCCAGGT TCCAACATAG TCATCCCTTC CAT
CTCCTTCTAC AGTGGGACCA TTGAGGTCCA AGGTTGTATC AGTAGGGAAG GTA
                                  **************************
```

Fragment 172/3.8

Lenght of the fragment = 840 bp

Arbitrary primer used : 5′ATG GAA GGG ATG ACT ATG TTG GA 3′

```
        10          20          30          40          50          60
*************************
ATGGAAGVBA TGACTATGTT GGAGTAGGTG TGGCCTTGCT GGAAGAAGTG CGTCACTGTG
TACCTTCBVT ACTGATACAA CCTCATCCAC ACCGGAACGA CCTTCTTCAC GCAGTGACAC

        70          80          90         100         110         120
AGGGCAGGCT TTAAGGTCTC ATACTCTCAA GCTACAGCCA GCGTGACAGT CTCTTTCTGC
TCCCGTCCGA AATTCCAGAG TATGAGAGTT CGATGTCGGT CGCACTGTCA GAGAAAGACG

       130         140         150         160         170         180
                               ************************
TGCCTGCAGA TCAAGATGTA GAACTCTATG GAAGGGATGA CTATGTTGGA GTAGGTGTGG
ACGGACGTCT AGTTCTACAT CTTGAGATAC CTTCCCTACT GATACAACCT CATCCACACC

       190         200         210         220         230         240
CCTTGCTGGA AGAAGTGCGT CACTGTGAGG GCAGGCTTTA AGGTCTCATA CTCTCAAGCT
GGAACGACCT TCTTCACGCA GTGACACTCC CGTCCGAAAT TCCAGAGTAT GAGAGTTCGA

       250         260         270         280         290         300
ACAGCCAGCG TGACAGTCTC TTTCTGCTGC CTGCAGATCA AGATGTAGAA CTCTCAGCTC
TGTCGGTCGC ACTGTCAGAG AAAGACGACG GACGTCTAGT TCTACATCTT GAGAGTCGAG

       310         320         330         340         350         360
CTTCTCTAGC ACCATGTCTG CCGGCATGCC ACCATGCTTC CCACCATGAT GATAATGGAC
GAAGAGATCG TGGTACAGAC GGCCGTACGG TGGTACGAAG GGTGGTACTA CTATTACCTG

       370         380         390         400         410         420
TGAACCTCTG AACCTGTAAG CCTGHGCCAG TAAAATGTTT TCCATTATAA GAGTTGTCAT
ACTTGGAGAC TTGGACATTC GGACDCGGTC ATTTTACAAA AGGTAATATT CTCAACAGTA

       430         440         450         460         470         480
GGTCATRGTG TCTCTYCAGA GCAGTAAAAC CCTCACTAAG AGAGTTGTGT TGCATGGATC
CCAGTAYCAC AGAGARGTCT CGTCATTTTG GGAGTGATTC TCTCAACACA ACGTACCTAG

       490         500         510         520         530         540
GGGACTGTGG CTCACTTAGG AGGTCATGAG GAAGATGTCA CCCTGGTAAC TCCAGGTTCC
CCCTGACACC GAGTGAATCC TCCAGTACTC CTTCTACAGT GGGACCATTG AGGTCCAAGG
                                                          ****
       550         560         570         580         590         600
AACATAGTCA TCCCTTCCAT TTGGAGACAT GCTGGAAAAA GCTGCCGAGC TGCTGATGAG
TTGTATCAGT AGGGAAGGTA AACCTCTGTA CGACCTTTTT CGACGGCTCG ACGACTACTC
**********************
       610         620         630         640         650         660
CTGCTTCCGA GTCTGTGCCA GTGACACGTG AGTGAGKTCT GGGGGCAACG TGCTCACCGT
GACGAAGGCT CAGACACGGT CACTGTGCAC TCACTCMAGA CCCCCGTTGC ACGAGTGGCA

       670         680         690         700         710         720
CAGAGCATGT GTTGCTCTCC CTGTACTCGG TCCTTGGTGC CGGCCACACT TTCTGCCTTC
GTCTCGTACA CAACGAGAGG GACATGAGCC AGGAACCACG GCCGGTGTGA AAGACGGAAG

       730         740         750         760         770         780
ACTTTGGAAG GTCTCTGCCC AGTGCACGCA GCCTGGCTTT CTCTACCTGT CAGTGCTCTG
TGAAACCTTC CAGAGACGGG TCACGTGCGT CGGACCGAAA GAGATGGACA GTCACGAGAC
```

```
       790         800         810         820         830         840
AGCAGTGCCC TTGTCTGGCT TTGACAGCCA GGCCCCCTCC AACATAGTCA TCVNTTCCAT
TCGTCACGGG AACAGACCGA AACTGTCGGT CCGGGGGAGG TTGTATCAGT AGBNAAGGTA
                                  **************************
```

Fragment 172/4.1

Length of the fragment =197 bp

Arbitrary primer used : 5′ATG GAA GGG ATG ACT ATG TTG GA 3′

```
        10          20          30          40          50          60
**************************--------------------->
ATGGAAGGGA TGACTATGTT GGAAGAGGAG GCGGCTGCGG AAAGGGTCTG GGTCTGGGTT
TACCTTCCCT ACTGATACAA CCTTCTCCTC CGCCGACGCC TTTCCCAGAC CCAGACCCAA

        70          80          90         100         110         120
TGCAGATTAG GACGACAAGG AGTTGGTTAC AAGGCCTGGT GAAGAGCTAG CTGGATAGGA
ACGTCTAATC CTGCTGTTCC TCAACCAATG TTCCGGACCA CTTCTCGATC GACCTATCCT

       130         140         150         160         170         180
TCGGTGGCCA TCCTGACTCA GGCCAGCTTC AGGAACTCCT GCAGTGTGTC CTGCTCCAAC
AGCCACCGGT AGGACTGAGT CCGGTCGAAG TCCTTGAGGA CGTCACACAG GACGAGGTTG
                <----------------------                  ******
       190         200         210         220         230         240
ATAGTCATCC CTTCCAT
TATCAGTAGG GAAGGTA
******************
```

Fragment 172/4.6 or 172/5.8

Length of the fragment = 397 bp

Arbitrary primer used : 5′ATG GAA GGG ATG ACT ATG TTG GA 3′

```
        10          20          30          40          50          60
**************************              ---------------------->
ATGGAAGGGA TGACTATGTT GGACTAGGTG TGCCACTGTG GGAGTGGGCT TTGAGACCCT
TACCTTCCCT ACTGATACAA CCTGATCCAC ACGGTGACAC CCTCACCCGA AACTCTGGGA

        70          80          90         100         110         120
CCTTCTAGCC GGGTGGGAND DAGCCCAGTC TTCTTCTGGG TTCCCTTGAA TAAGACATGG
GGAAGATCGG CCCACCCTNH HTCGGGTCAG AAGAAGACCC AAGGGAACTT ATTCTGTACC

       130         140         150         160         170         180
AACTCTCAGC TCCTTCTCCA GTGCCATGCC CGCCTGGATG CTGCCATGCT TCCCACCTTG
TTGAGAGTCG AGGAAGAGGT CACGGTACGG GCGGACCTAC GACGGTACGA AGGGTGGAAC

       190         200         210         220         230         240
ATGATAATGG ACTGAACCTC AGAACCAGTA AGCAGCCACA GTGAAATGCT GCCTTGGTCA
TACTATTACC TGACTTGGAG TCTTGGTCAT TCGTCGGTGT CACTTTACGA CGGAACCAGT

       250         260         270         280         290         300
TGGTGTCTGT TCACAGCAAT GGAACCCTAA GACAAGGTTC TTAACCCACA GGTATACCTT
ACCACAGACA AGTGTCGTTA CCTTGGGATT CTGTTCCAAG AATTGGGTGT CCATATGGAA

       310         320         330         340         350         360
CTGTCATCTG AACACTCACC AACTTTGACC ACTGTGACTG STTCCCCCAT TTCAGAGCCT
GACAGTAGAC TTGTGAGTGG TTGAAACTGG TGACACTGAC SAAGGGGGTA AAGTCTCGGA
                                             <----------------
       370         380         390         400         410         420
TTTCTCCTAG GVSCYMCAAC ATAGTCAYCC CTYCCAT
AAAGAGGATC CBSGRKGTTG TATCAGTRGG GARGGTA
----          ************************
```

Fragment 172/5.2

Length of the primer = 324 bp

Arbitrary primer used : 5′ATG GAA GGG ATG ACT ATG TTG GA 3′

```
        10          20          30          40          50          60
**************************                              ----
ATGGAAGGGA TGACTATGTT GGAGCTGGAA CTTCCCATGT AGACTATGGT AGTTTAGAAA
TACCTTCCCT ACTGATACAA CCTCGACCTT GAAGGGTACA TCTGATACCA TCAAATCTTT

        70          80          90         100         110         120
------------------>
CCACAGAGAT CTAACCGGCT TTGCCTCCCA GATCCTAAYY CTAAAGGGSG ACCCCACCCT
GGTGTCTCTA GATTGGCCGA AACGGAGGGT CTAGGATTRR GATTTCCCSC TGGGGTGGGA

       130         140         150         160         170         180
ATCCCTGCTC CCTTTTCTCT CTTAGCTTCC TTTATCAACC CAAGACCGAT CTTCCCACAG
TAGGGACGAG GGAAAAGAGA GAATCGAAGG AAATAGTTGG GTTCTGGCTA GAAGGGTGTC

       190         200         210         220         230         240
GATGGAGCAC AGGGATTGAC GAGTGACACT TGTCTGTACA GAGATTCCGA GGCCTTGAGT
CTACCTCGTG TCCCTAACTG CTCACTGTGA ACAGACATGT CTCTAAGGCT CCGGAACTCA

       250         260         270         280         290         300
TTGGGAATCT TCACCTAGGT AATACACTGC TCTTCCCCCT CCAACTCCCA CTCTCTCCCC
AACCCTTAGA AGTGGATCCA TTATGTGACG AGAAGGGGGA GGTTGAGGGT GAGAGAGGGG
                  <----------------------
       310         320         330         340         350         360
CTCCAACATA GTCATCCCTT CCAT
GAGGTTGTAT CAGTAGGGAA GGTA
**************************
```

Fragment 172/5.6

Length of the primer used = 659 bp

Arbitrary primer used : 5′ATG GAA GGG ATG ACT ATG TTG GA 3′

```
        10          20          30          40          50          60
**************************                  ------------------->
ATGGAAGGGA TGACTATGTT GGATTAAACA TGCATCACTG TGGAGGCAGG GCTTTGGGGA
TACCTTCCCT ACTGATACAA CCTAATTTGT ACGTAGTGAC ACCTCCGTCC CGAAACCCCT

        70          80          90          100         110         120
AATGTATGTA TACTCAGGCT CTGCCAAAGG TGAGACACAG TAATCTCATT TTCTGCCTTT
TTACATACAT ATGAGTCCGA GACGGTTTCC ACTCTGTGTC ATTAGAGTAA AAGACGGAAA

       130         140         150         160         170         180
GAATTGGGAG AGGGAGGCAA GCAGTTATCT GATATAGAAA ATCCTAGTCT GTAGAGAGAG
CTTAACCCTC TCCCTCCGTT CGTCAATAGA CTATATCTTT TAGGATCAGA CATCTCTCTC

       190         200         210         220         230         240
TTCCAGGGCA GCCTGGAGTA CACAGAAAAG CCCTATCTCA GAAAGAAATA ATGATCTTTC
AAGGTCCCGT CGGACCTCAT GTGTCTTTTC GGGATAGAGT CTTTCTTTAT TACTAGAAAG

       250         260         270         280         290         300
TTACTTGTGT CTTAGTCCTC CTTCAAAGAA GATGGTACTC ACCAGGTAGG TTGTTGATTC
AATGAACACA GAATCAGGAG GAAGTTTCTT CTACCATGAG TGGTCCATCC AACAACTAAG

       310         320         330         340         350         360
AGTTTCTGGT TATGGAATGG TCTTGGAGTA CAAATATTTT GCATTTTTTT TTACGGGGAT
TCAAAGACCA ATACCTTACC AGAACCTCAT GTTTATAAAA CGTAAAAAAA AATGCCCCTA

       370         380         390         400         410         420
TGCTCAAGCT TTCACATCTG ATAGAGGATC CATGTTTAAG ATCTGTGCCA ACCAGTGCCA
ACGAGTTCGA AAGTGTAGAC TATCTCCTAG GTACAAATTC TAGACACGGT TGGTCACGGT

       430         440         450         460         470         480
GCCATGATAC AMGCCATGAT TGTGAGACTC CTGCTGGTTT CCAAAGGGCC ACTGTAGTTG
CGGTACTATG TKCGGTACTA ACACTCTGAG GACGACCAAA GGTTTCCCGG TGACATCAAC

       490         500         510         520         530         540
GATGATGGTG GCATACAAAT GGATCACCCC ATACATGTGT TTTAGAAAGT TGTCCTTTTG
CTACTACCAC CGTATGTTTA CCTAGTGGGG TATGTACACA AAATCTTTCA ACAGGAAAAC
                          <-----------------------
       550         560         570         580         590         600
TTCAACCTTA GAATCCATCA CTTGGTAGAC AAGTATCCTT TGAGAATCCA ACATAGTCAT
AAGTTGGAAT CTTAGGTAGT GAACCATCTG TTCATAGGAA ACTCTTAGGT TGTATCAGTA
                                                ***************
       610         620         630         640         650         660
CCCTTCCAT
GGGAAGGTA
*********
```

Fragment 228/5.1

Length of fragment = estimated to be 316 bp (Table 4.3)

Arbitrary primer used : 5′ AGA AGT GAA GGC TGG CAT GGC 3′

```
        10          20          30          40          50          60
******************
AGTGAAGGCT GGCATGGCGA ACAGAAGGGA AACTCTGGTG GAGGTCCGTA GCGGTCCTGA
TCACTTCCGA CCGTACCGCT TGTCTTCCCT TTGAGACCAC CTCCAGGCAT CGCCAGGACT

        70          80          90         100         110         120
CGTGCAAATC GGTCGTCCGA CCTGGGTATA GGGGCGAAAG ACTAATCGAA CCATCTAGTA
GCACGTTTAG CCAGCAGGCT GGACCCATAT CCCCGCTTTC TGATTAGCTT GGTAGATCAT

       130         140         150         160         170         180
GCTGGTTCCC TCCGAAGTTT CCCTCAGGAT AGCTGGCGCY CTCGCTCTTT TTTTTTCCC-
CGACCAAGGG AGGCTTCAAA GGGAGTCCTA TCGACCGCGR GAGCGAGAAA AAAAAAGGG-

       190         200         210         220         230         240
---------GAP--------- TTTTTTTTTC CCGACGCTCA TCAGACCCCA GAAAAGGTGT
---------GAP--------- AAAAAAAAAG GGCTGCGAGT AGTCTGGGGT CTTTTCCACA

       250         260         270         280         290         300
TGGTTGATAT AGACAGCAGG ACGGTGGCCA TGCCAGCCTT CACT
ACCAACTATA TCTGTCGTCC TGCCACCGGT ACGGTCGGAA GTGA
```

Fragment 228/5.2

Length of the fragment = 340 bp

Arbitrary primer used : 5′ AGA AGT GAA GGC TGG CAT GGC 3′

```
        10          20          30          40          50          60
**********************                                  ------
AGAAGTGAAG GCTGGCATGG CGCCTCCAAA TCCTTTCACT TTCTGGAACT TCTTTTCTGG
TCTTCACTTC CGACCGTACC GCGGAGGTTT AGGAAAGTGA AAGACCTTGA AGAAAAGACC

        70          80          90         100         110         120
-------------->
CTGCAAAGTG AGTAGTAACC CTGTGCCAGT GCGATTGGCC TGGATGGCCC CGACACTCAG
GACGTTTCAC TCATCATTGG GACACGGTCA CGCTAACCGG ACCTACCGGG GCTGTGAGTC

       130         140         150         160         170         180
CTCCATCCGA CGTCCACGTC GAGTGCYCTC GTAACGGCTG AAGGTACCCA GAGCCGGTAA
GAGGTAGGCT GCAGGTGCAG CTCACGRGAG CATTGCCGAC TTCCATGGGT CTCGGCCATT

       190         200         210         220         230         240
GGTCACGGGG TCAAGAGAGT CACAGTACGA TGCATTACAG ACACAGACCA CTGAGCTGTA
CCAGTGCCCC AGTTCTCTCA GTGTCATGCT ACGTAATGTC TGTGTCTGGT GACTCGACAT

       250         260         270         280         290         300
GCCAAAGCTT TTGGGGATGC AGGGTTGGGC ACCATATGCC CAAGATACCG CCTGAAATAG
CGGTTTCGAA AACCCCTACG TCCCAACCCG TGGTATACGG GTTCTATGGC GGACTTTATC
       <----------------------

       310         320         330         340         350         360
GAAGAATCCG ATGAGCCTGG CAGCCATGCC AGCCTTCACT
CTTCTTAGGC TACTCGGACC GTCGGTACGG TCGGAAGTGA
                      ********************
```

Fragment 228/5.3

Length of the fragment = 301 bp

Arbitrary primer used : 5′AGA AGT GAA GGC TGG CAT GGC 3′

```
        10          20          30          40          50          60
**********************                                  -------
AGAAGTGAAG GCTGGCATGG CGAASCTCAG AGAGTCGTGT AGACCACAGC AGCCTCGCTA
TCTTCACTTC CGACCGTACC GCTTSGAGTC TCTCAGCACA TCTGGTGTCG TCGGAGCGAT

        70          80          90         100         110         120
---------------->
AGATGTCGGA AAAAAAGCCA AAAAGGATAA AGACCCAGTA AATAAATCTG GTGGCAAGGC
TCTACAGCCT TTTTTTCGGT TTTTCCTATT TCTGGGTCAT TTATTTAGAC CACCGTTCCG

       130         140         150         160         170         180
CAAGAAGAAG AAGTGGTCCA AAGGCAAGGT TCGGGACAAG TTGAATAATC TTGTCCTGTT
GTTCTTCTTC TTCACCAGGT TTCCGTTCCA AGCCCTGTTC AACTTATTAG AACAGGACAA

       190         200         210         220         230         240
CGACAAAGTG ACATACGACA AGCTCTGTAA GGAGGTTCCG AACTATAAGC TCATTACTCC
GCTGTTTCAC TGTATGCTGT TCGAGACATT CCTCCAAGGC TTGATATTCG AGTAATGAGG

       250         260         270         280         290         300
AGCCGTGGTC TCTGAGAGAC TGAAGATTCG CGGTTCCTTG GCCATGCCAG CCTTCACTTC
TCGGCACCAG AGACTCTCTG ACTTCTAAGC GCCAAGGAAC CGGTACGGTC GGAAGTGAAG
             <----------------------      **********************

       310         320         330         340         350         360
T
A
*
```

Fragment 228/5.6

Length of the fragment = 300 bp

Arbitrary primer used : 5′ AGA AGT GAA GGC TGG CAT GGC 3′

```
        10          20          30          40          50          60
**************************      ----------------------->
GTGAAGGGCT GGCATKGCCA TGCCAAGGTC CATCTGGTTG GCATTGACAT TTTTACTGGG
CACTTCCCGA CCGTAMCGGT ACGGTTCCAG GTAGACCAAC CGTAACTGTA AAAATGACCC

        70          80          90         100         110         120
AAGAAATATG AAGATATCTG CCCGTCAACT CATAATATGG ATGTCCCCAA CATCAAACGG
TTCTTTATAC TTCTATAGAC GGGCAGTTGA GTATTATACC TACAGGGGTT GTAGTTTGCC

       130         140         150         160         170         180
AATGACTTCC AGCTGATTGG CATCCAGGAT GGGTACCTAT CCCTGCTCCA GGACAGTGGG
TTACTGAAGG TCGACTAACC GTAGGTCCTA CCCATGGATA GGGACGAGGT CCTGTCACCC

       190         200         210         220         230         240
GAGGTACGAG AGGACCTTCG TCTGCCTGAA GGAGACCTTG GCAAGGAGAT TGAGCAGAAG
CTCCATGCTC TCCTGGAAGC AGACGGACTT CCTCTGGAAC CGTTCCTCTA ACTCGTCTTC
                                                              <-

       250         260         270         280         290         300
TATGACTGTG GAGAAGAGAT CCTGATCACA GTGCTGTCTG CCATGCCAGC CTTCACTTCT
ATACTGACAC CTCTTCTCTA GGACTAGTGT CACGACAGAC GGTACGGTCG GAAGTGAAGA
----------------------                  **********************
```

Some of the sequences of the fragments were found to be related after analysed by Contig Manager of the software DNAsis and by visual examiniations. In particular, fragment 172/4.6 and 172/5.8 represented exactly the same sequence (Figure 4.4, A). Fragment 172/2.8, 172/3.1, 172/3.2 and 172/3.8 were found to have a common stretch of sequence (Figure 4.4, B). Base no. 115-388 of fragment 172/2.8, 119-393 of fragment 172/3.1, 141-413 of fragment 172/3.2 and 287-560 of fragment 172/3.8 represented the same DNA sequences (Figure 4.4, B). For fragment 172/2.8, a third stretch of arbitrary primer sequences was found at base no. 365-388 besides the two franking the sequences. For fragment 172/3.8, two additional arbitrary sequence were found at base 147-170 and 536-560 (see above).

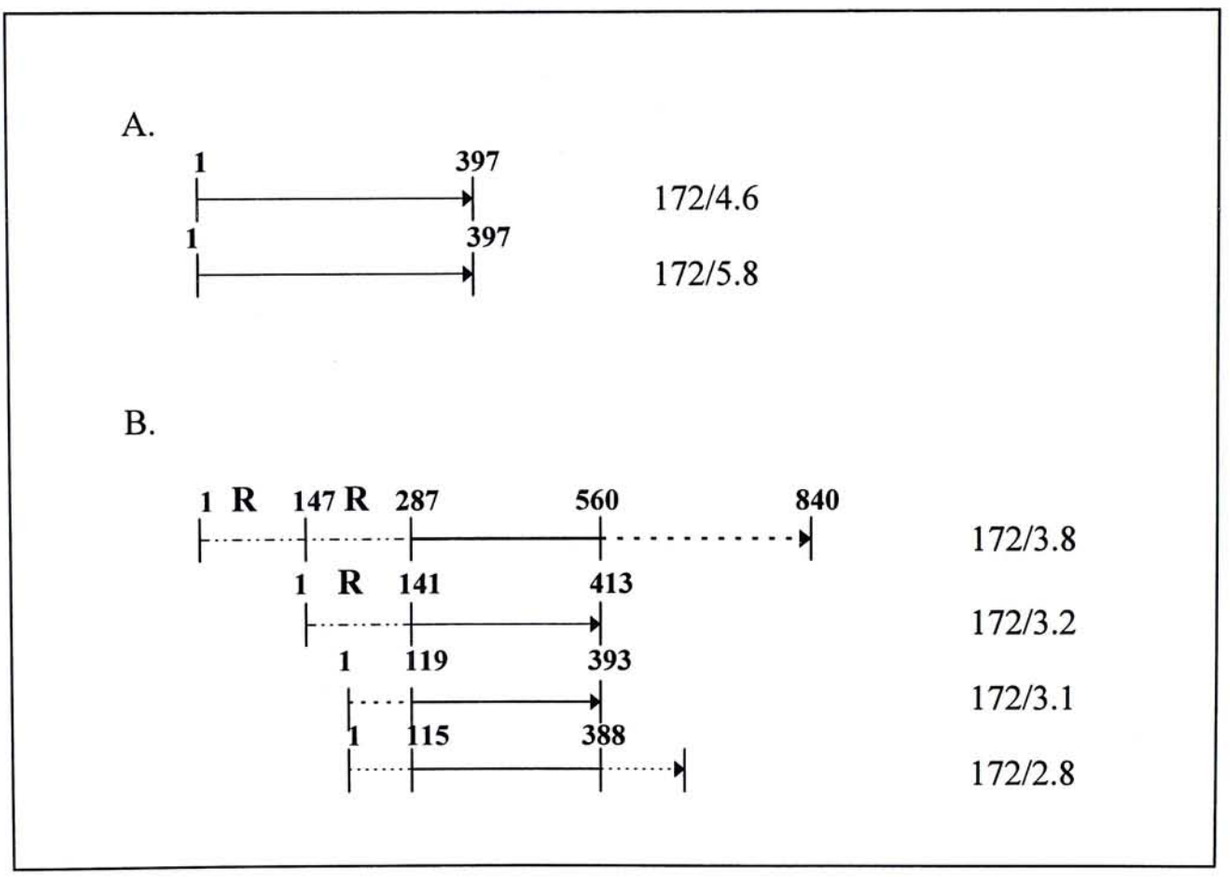


Figure 4.4. **Fragment analysis by DNAsis Contig Manager.** A. The sequence of 172/4.6 and 172/5.8 were found to be identical. B. Fragment 172/2.8, 172/3.2, 172/3.1 and 172/3.8 were found to be related sequences. ——— indicates identical sequence, ... indicates unique sequence. R is a stretch of sequence that happened twice in 172/3.8 and once in 172.3.2. The numbers on top of the lines indicate the base number (section 4.4.2).

4.4.3 BLASTN search result

By feeding the fragment sequence in ASC II format into the BLASTN program. Four main sections of results were generated. The first section was a histogram reporting the number of database sequences satisfying various expectation thresholds (i.e. the E or expect parameter). The second section was a hit list showing a summary of the database sequence that satisfies the expect parameter (i.e. 0.75 in our search). This section contained 7 items including the database designation, accession number, locus name, description of the sequence, alignment score, *p* value (probability) and the number of high-scoring segment pair (HSP). The third section showed the actual alignment; the number of alignments shown was governed by the parameter V. The final section was a summary of all the search parameters and query details. The following is a typical search having significant matches with some of the sequences in the database (Figure 4.5). The example was done by using the fragment sequence of 228/5.2. A summary of the matching result among all the fed-in sequences was tabulated (Table 4.4). Only 5 out of the 17 sequences were matched significantly with known sequences in the database. The exact alignments in the BLASTN program were shown in Figure 4.5 and 4.6.

A.

```
Query=  228-5-2b.seq
        (340 letters)

Database:  Non-redundant GenBank+EMBL+DDBJ+PDB sequences
           230,088 sequences; 314,684,217 total letters.
Searching.....................................................done


    Observed Numbers of Database Sequences Satisfying
   Various EXPECTation Thresholds (E parameter values)

       Histogram units:      = 4 Sequences      : less than 4 sequences

 EXPECTation Threshold
 (E parameter)
    |
    V   Observed Counts-->
 10000 652 214 |==========================================================
  6310 438 170 |=============================================
  3980 268  77 |====================
  2510 191  63 |================
  1580 128  23 |=====
  1000 105  30 |=======
   631  75  15 |===
   398  60  12 |===
   251  48   9 |==
   158  39   9 |==
   100  30   3 |:
  63.1  27   9 |==
  39.8  18   2 |:
  25.1  16   2 |:
  15.8  14   2 |:
  10.0  12   0 |
  6.31  12   1 |:
```

B.

Sequences producing	High-scoring Segment Pairs:	High Score	Smallest Sum Probability P(N)	N
gb|M24119|MUSGCB	Mouse glucocerebrosidase mRNA, comple...	1593	2.2e-125	1
gb|K02920|HUMGCBL	Human lysosomal glucocerebrosidase mR...	1086	5.2e-83	1
dbj|D13286|HUMGCA	Human mRNA for glucocerebrosidase, co...	1086	5.7e-83	1
gb|M19285|HUMGCBPRC	Human glucocerebrosidase mRNA, comple...	1077	3.2e-82	1
gb|M16328|HUMGCB	Human glucocerebrosidase mRNA, comple...	1077	3.3e-82	1
gb|I09351|	Sequence 1 from Patent WO 8905850	1005	2.9e-76	1
gb|J03059|HUMGCB1	Human glucocerebrosidase (GCB) gene, ...	678	1.2e-66	2
gb|J03060|HUMGCB2	Human glucocerebrosidase pseudogene, ...	258	4.3e-35	4
gb|M89949|MUSGBA	BALB/c glucocerebrosidase (Gba) gene,...	302	2.3e-15	1
gb|M20248|HUMGCBDA	Human glucocerebrosidase gene, 5' end.	231	6.3e-10	1
dbj|D13287|HUMGCBA	Human mRNA for alternative splicing p...	172	0.00024	1

C.

```
>gb|M24119|MUSGCB Mouse glucocerebrosidase mRNA, complete cds.
           Length = 1684

  Minus Strand HSPs:

 Score = 1593 (440.8 bits), Expect = 2.2e-125, P = 2.2e-125
 Identities = 324/332 (97%), Positives = 325/332 (97%), Strand = Minus / Plus

Query:   333 GCTGGCATGGCTGCCAGGCTCATCGGATTCTTCCTATTTCAGGCGGTATCTTGGGCATAT 274
             | || ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:    81 GGTGTCATGGCTGCCAGGCTCATCGGATTCTTCCTATTTCAGGCGGTATCTTGGGCATAT 140

Query:   273 GGTGCCCAACCCTGCATCCCCAAAAGCTTTGGCTACAGCTCAGTGGTCTGTGTCTGTAAT 214
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   141 GGTGCCCAACCCTGCATCCCCAAAAGCTTTGGCTACAGCTCAGTGGTCTGTGTCTGTAAT 200

Query:   213 GCATCGTACTGTGACTCTCTTGACCCCGTGACCTTACCGGCTCTGGGTACCTTCAGCCGT 154
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   201 GCATCGTACTGTGACTCTCTTGACCCCGTGACCTTACCGGCTCTGGGTACCTTCAGCCGT 260

Query:   153 TACGAGRGCACTCGACGTGGACGTCGGATGGAGCTGAGTGTCGGGGCCATCCAGGCCAAT 94
             ||||||+||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   261 TACGAGAGCACTCGACGTGGACGTCGGATGGAGCTGAGTGTCGGGGCCATCCAGGCCAAT 320

Query:    93 CGCACTGGCACAGGGTTACTACTCACTTTGCAGCCAGAAAAGAAGTTCCAGAAAGTGAAA 34
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   321 CGCACTGGCACAGGGTTACTACTCACTTTGCAGCCAGAAAAGAAGTTCCAGAAAGTGAAA 380

Query:    33 GGATTTGGAGGCGCCATGCCAGCCTTCACTTC 2
             |||||||||||||||||| ||| |  |||| |
Sbjct:   381 GGATTTGGAGGCGCCATGACAGACGCCACTGC 412


>gb|K02920|HUMGCBL Human lysosomal glucocerebrosidase mRNA, complete cds.
           Length = 1792

  Minus Strand HSPs:

 Score = 1086 (300.5 bits), Expect = 5.2e-83, P = 5.2e-83
 Identities = 261/317 (82%), Positives = 262/317 (82%), Strand = Minus / Plus

Query:   328 CATGGCTGCCAGGCTCATCGGATTCTTCCTATTTCAGGCGGTATCTTGGGCATATGGTGC 269
             |||||||| ||| ||||  |||||  | ||| ||||||| || || |||||||  |||||
Sbjct:    45 CATGGCTGGCAGCCTCACAGGATTGCTTCTACTTCAGGCAGTGTCGTGGGCATCAGGTGC 104

Query:   268 CCAACCCTGCATCCCCAAAAGCTTTGGCTACAGCTCAGTGGTCTGTGTCTGTAATGCATC 209
             ||  ||||||||||| |||||||| |||||||||| ||||| |||||||| |||||  |
Sbjct:   105 CCGCCCCTGCATCCCTAAAAGCTTCGGCTACAGCTCGGTGGTGTGTGTCTGCAATGCCAC 164

Query:   208 GTACTGTGACTCTCTTGACCCCGTGACCTTACCGGCTCTGGGTACCTTCAGCCGTTACGA 149
              ||||||||||  ||||||||  |||||| || || || ||||||||||||| || ||
Sbjct:   165 ATACTGTGACTCCTTTGACCCCCCGACCTTTCCTGCCCTTGGTACCTTCAGCCGCTATGA 224

Query:   148 GRGCACTCGACGTGGACGTCGGATGGAGCTGAGTGTCGGGGCCATCCAGGCCAATCGCAC 89
             |+| || ||  |||| || ||||||||||||| | ||| |||||||||| |||| |||
Sbjct:   225 GAGTACACGCAGTGGGCGACGGATGGAGCTGAGTATGGGGCCCATCCAGGCTAATCACAC 284

Query:    88 TGGCACAGGGTTACTACTCACTTTGCAGCCAGAAAAGAAGTTCCAGAAAGTGAAAGGATT 29
              ||||||||  | ||||| ||  |||||||||| |||||||||||||||||| |||||
Sbjct:   285 GGGCACAGGCCTGCTACTGACCCTGCAGCCAGAACAGAAGTTCCAGAAAGTGAAGGGATT 344

Query:    28 TGGAGGCGCCATGCCAG 12
             |||||| |||||| |||
Sbjct:   345 TGGAGGGGCCATGACAG 361
```

D.

```
Parameters:
  V=100
  B=50
  H=1
  -qtype
  E=0.75
  P=4

  -ctxfactor=2.00

  Query                          -----  As Used  -----     -----  Computed  ----
  Strand MatID Matrix name       Lambda     K        H      Lambda     K        H
   +1      0   +5,-4             0.192   0.173    0.357    same     same     same
   -1      0   +5,-4             0.192   0.173    0.357    same     same     same

  Query
  Strand MatID  Length  Eff.Length   E      S  W   T  X      E2  S2
   +1      0      340        340     0.75 128 11 N/A 73   0.023 76
   -1      0      340        340     0.75 128 11 N/A 73   0.023 76

Statistics:
  Query           Expected           Observed           HSPs       HSPs
  Strand MatID  High Score         High Score        Reportable  Reported
   +1      0    122 (33.8 bits)    120 (33.2 bits)       0          0
   -1      0    122 (33.8 bits)   1593 (440.8 bits)     16         16

  Query          Neighborhd  Word      Excluded    Failed   Successful  Overlaps
  Strand MatID    Words      Hits        Hits    Extensions Extensions  Excluded
   +1      0        325       24262       1955      21486      821         0
   -1      0        325       26406       2265      23259      882         0

  Database:  Non-redundant GenBank+EMBL+DDBJ+PDB sequences
    Release date:  March 22, 1996
    Posted date:  3:31 AM EST Mar 22, 1996
  # of letters in database:  314,684,217
  # of sequences in database:  230,088
  # of database sequences satisfying E:  11
  No. of states in DFA:  224 (224 KB)
  Total size of DFA:  232 KB (256 KB)
  Time to generate neighborhood:  0.02u 0.01s 0.03t  Real: 00:00:00
  No. of processors used:  4
  Time to search database:  20.07u 2.19s 22.26t  Real: 00:00:09
  Total cpu time:  20.11u 2.23s 22.34t  Real: 00:00:09
```

Figure 4.5. **A typical BLASTN search result demonstrated by fragment 228/5.2.** The results had four parts, namely, A. histogram of number of database sequences; B. hit list; C. alignments of the query with the database sequence; D. the search details (see also text).

A. Fragment 44/3.1 (Length estimated to be 870 bp, Table 4.3)

```
>gb|M22664|MUSMTREPA Mouse MT repetitive element DNA.
           Length = 400

  Minus Strand HSPs:

 Score = 1183 (328.6 bits), Expect = 1.5e-121, Sum P(2) = 1.5e-121
 Identities = 254/277 (91%), Positives = 255/277 (92%), Strand = Minus / Plus

Query:   662 TGTCTTAGTCAAGGTCTCTATTCCTGCACAAACATCATGACCAAGAAGCGAGTTGGGGAG 603
            |||||||||||  ||||||||||||||||||||||||||||||||||||  ||  |||||||
Sbjct:     2 TGTCTTAGTCAGGGTCTCTATTCCTGCACAAACATCATGACCAAGAAGCAAGCTGGGGAG 61

Query:   602 GAAAGGGTTTATTTGGCTTACACTTCCATGCTGCTGTTCATCACCAAAGGAAGTCAGGCC 543
            |||||||||||||  ||||||||||||||||||||||||||||||||||||||||||||| |
Sbjct:    62 GAAAGGGTTTATTCGGCTTACACTTCCATGCTGCTGTTCATCACCAAAGGAAGTCAGGAC 121

Query:   542 TGGAACTCAAGCAGGTCAGGAAGCAGGAGCTGATGCAGAGGCCATGAAGGGATGTTCCTT 483
            ||||||||||||||||||  |||||||||||||||||||||||||  |||||||||||||
Sbjct:   122 TGGAACTCAAGCAGGTCAGAAAGCAGGAGCTGATGCAGAGGCCATGGAGGGATGTTCCTT 181

Query:   482 ACTATCTTGCCTCCCCTGGCTTGCTCAGCCTGCTCTCTTATAGAACCCAAGACTGCCAGT 423
            ||||  |||||||||  |||||||||||||||||||||||||  |||  |||||||||||||
Sbjct:   182 ACTAGCTTGCCTCCTCTGGCTTGCTCAGCCTGCTCTCTTATGGAATCCAAGACTGCCAGC 241

Query:   422 CCAGAGATGGTCCCMCCCACAAGGGGCCTTTCCCCCT 386
            |||||||||  || +||   | |||  ||||  |||| |
Sbjct:   242 CCAGAGATGTCCCACCCACAAGGGGCCCTTCCCCCTT 278

 Score = 344 (95.6 bits), Expect = 1.5e-121, Sum P(2) = 1.5e-121
 Identities = 74/82 (90%), Positives = 74/82 (90%), Strand = Minus / Plus

Query:   413 GTCCCMCCCACAAGGGGCCTTTCCCCCTTGATCACTAATTGAGAAAATGCCTTACAGCTA 354
            |||||  |||||||||||||  |||||||||||||||||||||||||||||||||||||| |
Sbjct:   250 GTCCCACCCACAAGGGGCCCTTCCCCCTTGATCACTAATTGAGAAAATGCCTTACAGTTG 309

Query:   353 GATCTCATGGGGGBHTTTCCAC 332
            ||||||||||  ||  |||||  |
Sbjct:   310 GATCTCATGGAGGCATTTCCTC 331
```

B. Fragment 228/5.1 (Length estimated to be 316 bp, Table 4.3)

```
>emb|X00525|MMRNA02 Mouse 28S ribosomal RNA
           Length = 4712

  Plus Strand HSPs:

 Score = 727 (201.2 bits), Expect = 1.2e-74, Sum P(2) = 1.2e-74
 Identities = 150/157 (95%), Positives = 151/157 (96%), Strand = Plus / Plus

Query:    11 GGCATGGCGAACAGAAGGGAAACTCTGGTGGAGGTCCGTAGCGGTCCTGACGTGCAAATC 70
             |||| ||||||   | ||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  1356 GGCAGGGCGAAGCCAGAGGAAACTCTGGTGGAGGTCCGTAGCGGTCCTGACGTGCAAATC 1415


Query:    71 GGTCGTCCGACCTGGGTATAGGGGCGAAAGACTAATCGAACCATCTAGTAGCTGGTTCCC 130
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  1416 GGTCGTCCGACCTGGGTATAGGGGCGAAAGACTAATCGAACCATCTAGTAGCTGGTTCCC 1475

Query:   131 TCCGAAGTTTCCCTCAGGATAGCTGGCGCYCTCGCTC 167
             |||||||||||||||||||||||||||+|||||||
Sbjct:  1476 TCCGAAGTTTCCCTCAGGATAGCTGGCGCTCTCGCTC 1512

 Score = 314 (86.9 bits), Expect = 1.2e-74, Sum P(2) = 1.2e-74
 Identities = 66/70 (94%), Positives = 66/70 (94%), Strand = Plus / Plus

Query:   188 TCCCGACGCTCATCAGACCCCAGAAAAGGTGTTGGTTGATATAGACAGCAGGACGGTGGC 247
             | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  1719 TGCCGACGCTCATCAGACCCCAGAAAAGGTGTTGGTTGATATAGACAGCAGGACGGTGGC 1778

Query:   248 CATGCCAGCC 257
             ||||  || |
Sbjct:  1779 CATGGAAGTC 1788


>gb|M30952|ORARGITX Orangutan 28S ribosomal RNA gene fragment.
           Length = 2487

  Plus Strand HSPs:

 Score = 717 (198.5 bits), Expect = 3.5e-74, Sum P(2) = 3.5e-74
 Identities = 148/155 (95%), Positives = 149/155 (96%), Strand = Plus / Plus

Query:    11 GGCATGGCGAACAGAAGGGAAACTCTGGTGGAGGTCCGTAGCGGTCCTGACGTGCAAATC 70
             |||| ||||||   | ||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  1220 GGCAGGGCGAAGCCAGAGGAAACTCTGGTGGAGGTCCGTAGCGGTCCTGACGTGCAAATC 1279

Query:    71 GGTCGTCCGACCTGGGTATAGGGGCGAAAGACTAATCGAACCATCTAGTAGCTGGTTCCC 130
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  1280 GGTCGTCCGACCTGGGTATAGGGGCGAAAGACTAATCGAACCATCTAGTAGCTGGTTCCC 1339

Query:   131 TCCGAAGTTTCCCTCAGGATAGCTGGCGCYCTCGC 165
             |||||||||||||||||||||||||||+|||||
Sbjct:  1340 TCCGAAGTTTCCCTCAGGATAGCTGGCGCTCTCGC 1374

 Score = 314 (86.9 bits), Expect = 3.5e-74, Sum P(2) = 3.5e-74
 Identities = 66/70 (94%), Positives = 66/70 (94%), Strand = Plus / Plus

Query:   188 TCCCGACGCTCATCAGACCCCAGAAAAGGTGTTGGTTGATATAGACAGCAGGACGGTGGC 247
             | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  1590 TGCCGACGCTCATCAGACCCCAGAAAAGGTGTTGGTTGATATAGACAGCAGGACGGTGGC 1649

Query:   248 CATGCCAGCC 257
             ||||  || |
Sbjct:  1650 CATGGAAGTC 1659
```

C. Fragment 228/5.3 (Length = 301)

```
>emb|X62482|RRRPS25 R.rattus mRNA for ribosomal protein S25
           Length = 466

  Plus Strand HSPs:

 Score = 914 (252.9 bits), Expect = 3.4e-71, P = 3.4e-71
 Identities = 206/235 (87%), Positives = 206/235 (87%), Strand = Plus / Plus

Query:    60 AAGATGTCGGAAAAAAAGCCAAAAAGGATAAAGACCCAGTAAATAAATCTGGTGGCAAGG 119
             | |||| ||||||    |||||||| || || ||||||||||||||||||||||||||||
Sbjct:    68 AGGATGCCGGAAAGTCGGCCAAAAAAGACAAGGACCCAGTAAATAAATCTGGTGGCAAGG 127

Query:   120 CCAAGAAGAAGAAGTGGTCCAAAGGCAAGGTTCGGGACAAGTTGAATAATCTTGTCCTGT 179
             |||| |||||||||||||||||||||| |||||||||||| |||| ||||| |||||||
Sbjct:   128 CCAAAAAGAAGAAGTGGTCCAAAGGCAAAGTTCGGGACAAGCTGAACAATCTCGTCCTGT 187

Query:   180 TCGACAAAGTGACATACGACAAGCTCTGTAAGGAGGTTCCGAACTATAAGCTCATTACTC 239
             | |||||||  || |||||||| || |||||||| ||||| |||||||||||| |||||||
Sbjct:   188 TTGACAAAGCTACTTACGACAAACTTTGTAAGGAAGTTCCCAACTATAAGCTTATTACTC 247

Query:   240 CAGCCGTGGTCTCTGAGAGACTGAAGATTCGCGGTTCCTTGGCCATGCCAGCCTT 294
             |||| |||||||| |||||||||||||||| |||||||||||| | ||||| |
Sbjct:   248 CAGCTGTGGTCTCCGAGAGACTGAAGATTCGAGGTTCCTTGGCCAGGGCAGCCCT 302

  Minus Strand HSPs:

 Score = 172 (47.6 bits), Expect = 0.00017, P = 0.00017
 Identities = 40/47 (85%), Positives = 40/47 (85%), Strand = Minus / Plus

Query:    56 GAGGCTGCTGTGGTCTACACGACTCTCTGAGSTTCGCCATGCCAGCC 10
             | |||||| |||||| |||| |||||||||| |||||||||||  ||
Sbjct:     2 GTGGCTGCAGTGGTCCACACTACTCTCTGAGTTTCGCCATGCCGCCC 48


>gb|U10900|RNU10900 Rattus norvegicus Sprague-Dawley alcohol dehydrogenase
            class I (ADH3) gene, 5' region, and ribosomal protein S25
            pseudogene,  complete cds.
            Length = 3113

  Plus Strand HSPs:
 Score = 869 (240.5 bits), Expect = 7.5e-65, P = 7.5e-65
 Identities = 201/235 (85%), Positives = 201/235 (85%), Strand = Plus / Plus

Query:    60 AAGATGTCGGAAAAAAAGCCAAAAAGGATAAAGACCCAGTAAATAAATCTGGTGGCAAGG 119
             | |||  |||||||   |||||||| || ||||||||||||||||||||||||||||||
Sbjct:  2544 AGGATACCGGAAAATCGGCCAAAAAAGACAAAGACCCAGTAAATAAATCTGGTGGCAAGG 2603

Query:   120 CCAAGAAGAAGAAGTGGTCCAAAGGCAAGGTTCGGGACAAGTTGAATAATCTTGTCCTGT 179
             |||| |||||||||||||||||||||| ||| |||||||| |||| |||||||||| |
Sbjct:  2604 CCAAAAAGAAGAAGTGGTCCAAAGGCAAAGTTTGGGACAAGCTGAACAATCTTGTCCTAT 2663

Query:   180 TCGACAAAGTGACATACGACAAGCTCTGTAAGGAGGTTCCGAACTATAAGCTCATTACTC 239
             | |||||||  || || ||||| || |||||||| ||| | |||||||||||| |||||||
Sbjct:  2664 TTGACAAAGCTACTTAGGACAAACTTTGTAAGGAAGTTTCCAACTATAAGCTTATTACTC 2723

Query:   240 CAGCCGTGGTCTCTGAGAGACTGAAGATTCGCGGTTCCTTGGCCATGCCAGCCTT 294
             |||| |||||||| |||||||||||||||| ||||||||| || | |   |||
Sbjct:  2724 CAGCTGTGGTCTCCGAGAGACTGAAGATTCGAGGTTCCTTGGTCAGGACCTACTT 2778

  Minus Strand HSPs:

 Score = 201 (55.6 bits), Expect = 7.3e-07, P = 7.3e-07
 Identities = 48/59 (81%), Positives = 49/59 (83%), Strand = Minus / Plus

Query:    68 CGACATCTTAGCGAGGCTGCTGTGGTCTACACGACTCTCTGAGSTTCGCCATGCCAGCC 10
             |||||||||||  | || ||| |||||| |||| ||||| ||| +||||||||||  ||
Sbjct:  2466 CGACATCTTAGTGTGGTTGCAGTGGTCCACACTACTCTGTGAACTTCGCCATGCCGCCC 2524
```

D. Fragment 228/5.6 (Length = 300 bp)

```
>gb|M23419|HUMEIF4D Human initiation factor 4D 9eIF 4D) mRNA, complete cds.
           Length = 558

  Plus Strand HSPs:

 Score = 1287 (356.2 bits), Expect = 2.4e-102, P = 2.4e-102
 Identities = 270/287 (94%), Positives = 271/287 (94%), Strand = Plus / Plus

Query:      3 GAAGGGCTGGCATKGCCATGCCAAGGTCCATCTGGTTGGCATTGACATTTTTACTGGGAA 62
              ||  |||  ||| +|||| |||||||||||||||||||| |||||||| ||||||||||
Sbjct:    184 GACTGGCAAGCACGGCCACGCCAAGGTCCATCTGGTTGGTATTGACATCTTTACTGGGAA 243

Query:     63 GAAATATGAAGATATCTGCCCGTCAACTCATAATATGGATGTCCCCAACATCAAACGGAA 122
              |||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||
Sbjct:    244 GAAATATGAAGATATCTGCCCGTCAACTCATAATATGGATGTCCCCAACATCAAAAGGAA 303

Query:    123 TGACTTCCAGCTGATTGGCATCCAGGATGGGTACCTATCCCTGCTCCAGGACAGTGGGGA 182
              |||||||||||||||||||||||||||||||||||| |||||||||||||| |||||
Sbjct:    304 TGACTTCCAGCTGATTGGCATCCAGGATGGGTACCTATCACTGCTCCAGGACAGCGGGGA 363

Query:    183 GGTACGAGAGGACCTTCGTCTGCCTGAAGGAGACCTTGGCAAGGAGATTGAGCAGAAGTA 242
              |||||||||||||||||||| ||||| |||||||||||||||||||||||||||||||
Sbjct:    364 GGTACGAGAGGACCTTCGTCTCCCTGAGGGAGACCTTGGCAAGGAGATTGAGCAGAAGTA 423

Query:    243 TGACTGTGGAGAAGAGATCCTGATCACAGTGCTGTCTGCCATGCCAG 289
              ||||||||||||||||||||||||| ||||||||||||| |||
Sbjct:    424 CGACTGTGGAGAAGAGATCCTGATCACGGTGCTGTCTGCCATGACAG 470


>gb|S72024|S72024 eif-5A=eukaryotic initiation factor 5A {clone cos 9.1}
           [human, placenta, Genomic, 558 nt]
           Length = 558

  Plus Strand HSPs:

 Score = 1269 (351.2 bits), Expect = 7.5e-101, P = 7.5e-101
 Identities = 268/287 (93%), Positives = 269/287 (93%), Strand = Plus / Plus

Query:      3 GAAGGGCTGGCATKGCCATGCCAAGGTCCATCTGGTTGGCATTGACATTTTTACTGGGAA 62
              ||  |||  ||| +|||| |||||||||||||||||||| |||||||| ||||||||||
Sbjct:    184 GACTGGCAAGCACGGCCACGCCAAGGTCCATCTGGTTGGTATTGACATCTTTACTGGGAA 243

Query:     63 GAAATATGAAGATATCTGCCCGTCAACTCATAATATGGATGTCCCCAACATCAAACGGAA 122
              |||||||||||||||||||| |||||||||||||||||||||||||||| | ||||
Sbjct:    244 GAAATATGAAGATATCTGCCCATCAACTCATAATATGGATGTCCCCAACATCAGAAGGAA 303

Query:    123 TGACTTCCAGCTGATTGGCATCCAGGATGGGTACCTATCCCTGCTCCAGGACAGTGGGGA 182
              |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
Sbjct:    304 TGACTTCCAGCTGATTGGCATCCAGGATGGGTACCTATCACTGCTCCAGGACAGTGGGGA 363

Query:    183 GGTACGAGAGGACCTTCGTCTGCCTGAAGGAGACCTTGGCAAGGAGATTGAGCAGAAGTA 242
              ||||| ||||||||||||||| ||||| |||||||||||||||||||||||||||||||
Sbjct:    364 GGTACCAGAGGACCTTCGTCTCCCTGAGGGAGACCTTGGCAAGGAGATTGAGCAGAAGTA 423

Query:    243 TGACTGTGGAGAAGAGATCCTGATCACAGTGCTGTCTGCCATGCCAG 289
              ||||||||||||||||||||||||| ||||||||||||| |||
Sbjct:    424 CGACTGTGGAGAAGAGATCCTGATCACGGTGCTGTCTGCCATGACAG 470
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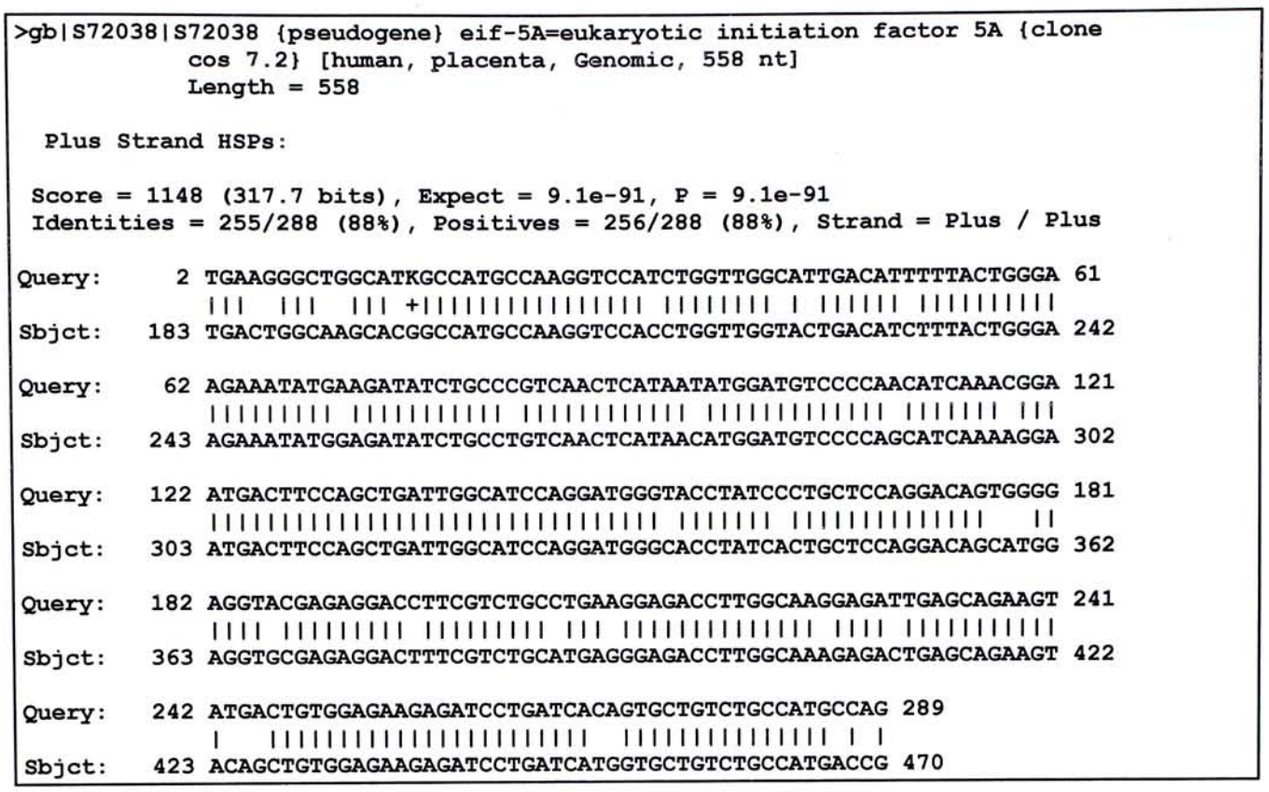


```
>gb|S72038|S72038 {pseudogene} eif-5A=eukaryotic initiation factor 5A {clone
           cos 7.2} [human, placenta, Genomic, 558 nt]
           Length = 558

  Plus Strand HSPs:

 Score = 1148 (317.7 bits), Expect = 9.1e-91, P = 9.1e-91
 Identities = 255/288 (88%), Positives = 256/288 (88%), Strand = Plus / Plus

Query:      2 TGAAGGGCTGGCATKGCCATGCCAAGGTCCATCTGGTTGGCATTGACATTTTTACTGGGA 61
              |||  |||  ||| +|||||||||||||||| |||||||| | ||||||  ||||||||||
Sbjct:    183 TGACTGGCAAGCACGGCCATGCCAAGGTCCACCTGGTTGGTACTGACATCTTTACTGGGA 242

Query:     62 AGAAATATGAAGATATCTGCCCGTCAACTCATAATATGGATGTCCCCAACATCAAACGGA 121
              ||||||||| |||||||||| |||||||||||| |||||||||||| ||||||| |||
Sbjct:    243 AGAAATATGGAGATATCTGCCTGTCAACTCATAACATGGATGTCCCCAGCATCAAAAGGA 302

Query:    122 ATGACTTCCAGCTGATTGGCATCCAGGATGGGTACCTATCCCTGCTCCAGGACAGTGGGG 181
              |||||||||||||||||||||||||||||| ||||||| |||||||||||||   ||
Sbjct:    303 ATGACTTCCAGCTGATTGGCATCCAGGATGGGCACCTATCACTGCTCCAGGACAGCATGG 362

Query:    182 AGGTACGAGAGGACCTTCGTCTGCCTGAAGGAGACCTTGGCAAGGAGATTGAGCAGAAGT 241
              |||| ||||||||| ||||||||| ||| |||||||||||||| |||| ||||||||||
Sbjct:    363 AGGTGCGAGAGGACTTTCGTCTGCATGAGGGAGACCTTGGCAAAGAGACTGAGCAGAAGT 422

Query:    242 ATGACTGTGGAGAAGAGATCCTGATCACAGTGCTGTCTGCCATGCCAG 289
              |   ||||||||||||||||||||||  |||||||||||||| | |
Sbjct:    423 ACAGCTGTGGAGAAGAGATCCTGATCATGGTGCTGTCTGCCATGACCG 470
```

Figure 4.6. **BLASTN search result (alignments) of fragment 44/3.1 (A), 228/5.1(B), 228/5.3(C) and 228/5.6 (D).** Only significant alignments were shown. In the case where the same genes or sequences of different species were matched with the query sequences, only the first two of the list were shown. See also Figure 4.5 for fragment 228/5.2.

Table 4.4. **Summary of matching results by feeding the fragment sequences into the BLASTN program.**

Gene fragment	Matching result
**44/3.1	MT sequence repetitive element DNA
**228/5.1	Mouse 28S ribosomal RNA Orangutan 28S ribosomal RNA gene fragment Chimpanzee 28S ribosomal RNA gene Human 28S ribosomal RNA gene
*228/5.2	Mouse glucocerebrosidase mRNA, complete cds. Human lysosomal glucocerebrosidase mRNA, complete cds. Human mRNA for glucocerebrosidase mRNA, complete cds.
**228/5.3	*R. rattus* mRNA for ribosomal protein S25 *Rattus norvegicus* Sprague-Dawley alcohol dehydrogenase Class I (ADH3) gene, 5'-region, and ribosomal protein S25 pseudogene, complete cds. Human ribosomal protein S25 mRNA, complete cds.
**228/5.6	Human initiation factor 4D (eIF 4D) mRNA, complete cds. eif-5A=eukaryotic initiation factor 5A{clone cos9.1} {pseudogene} eif-5A=eukaryotic initiation factor 5A {clone cos 7.2} [human, placenta, genomic, 558 nt]

Five of the gene fragments matched with known sequences in the Genbank (44/3.1, 228/5.1, 228/5.2, 228/5.3 and 228/5.6) were shown while others showed many insignificant matches (147/1.4, 172/2.8, 172/3.1, 172/3.2, 172/3.8, 172/4.6, 172/5.2, 172/5.6 and 172/5.8) or no matches (147/1.5, 147/1.6 and 172/4.1) at all under the current cutoff scores and parameters (see also Figure 4.5* and 4.6**).

4.4.4 Primer design of the sequenced fragments

Primers for RT-PCR (section 4.4.5) were designed with the help of the software OLIGO^{TM} (National BioSciences). False priming, internal stability, primer-dimer formation and self-complementarity were checked. The melting temperature of the primers were calculated from 2 °C (A+T) + 4 °C (G+C) as shown in Table 4.5. The expected product sizes were also shown. Gene fragments 228/5.1 matched with 28S ribosomal RNA were neglected for the moment.

Table 4.5. **Summary of designed primers based on sequencing result in section 4.4.2.**

Fragment no.		Sequence (5' to 3')	Tm (°C)*	Product size
44/3.1	upper primer 1	TAA ATG AGG TAA CTG ATG TGG GA	64	236
	lower primer 1	CTT AGT GAT CAG AGA AAT GCA AAT	64	
	upper primer 2	CCC CCA TGA GAT CTA GCT GTA	64	300
	lower primer 2	CCT GCA CAA ACA TCA TGA CCA A	64	
147/1.4	upper primer	CAT GTG GTT GCT GGG ATT TGA A	64	291
	lower primer	CAT GTG GTT GCT GGG ATT TGA A	64	
147/1.5	upper primer	CCC CCA AAA TCA GAG TAG ACA TA	66	>286
	lower primer	GCT TCT GGC TGC TCT CCC TC	66	
147/1.6	upper primer	AGC ACC ACT CTC AGG GGA ATC	66	222
	lower primer	TCC AAA CAG TAC TAC CCC ATG C	66	
228/5.2	upper primer	TTC TGG CTG CAA AGR GAG TAG TA	66	215
	lower primer	CCC AAC CCT GCA TCC CCA AAA	66	
228/5.3	upper primer	CTC GCT AAG ATG TCG GAA AAA AA	64	222
	lower primer	AAC CGC GAA TCT TCA GTC TCT C	66	
228/5.6	upper primer	CAT CTG GTT GGC ATT GAC ATT TTT	66	231
	lower primer	GAT CTC TTC TCC ACA GTC ATA CT	66	
172/3.2	upper primer	TTG CTG GAA GAA GTG CGT CAC	64	303
	lower primer	GTC CTG ATC CAT GCA ACA CAA	62	
172/4.1	upper primer	AGA GGA GGC GGC TGC GGA AA	66	135
	lower primer	GAG TTC CTG AAG CTG GCC TGA	66	
172/5.2	upper primer	GAA ACC ACA GAG ATC TAA CCG G	66	222
	lower primer	GGG GAA GAG CAG TGT ATT ACC T	66	
172/5.6	upper primer	TGG AGG CAG GGC TTT GGG G	64	488
	lower primer	TTT CTA AAA CAC ATG TAT GGG GT	62	
172/4.6	upper primer	CTG TGG GAG TGG GCT TTG AGA	66	329
172/5.8	lower primer	GAA AAG GCT CTG AAA TGG GGG	66	

*Tm was calculated from the formula 2 °C (A+T) + 4 °C (G+C)

These primer sequences were selected internal to the arbitrary primer sequence used in RAP-PCR. See also section 4.4.2 for the exact positions of these primers in the subcloned gene fragments.

4.4.5 Expression profile of isolated genes in midazolam-, biochanin A-induced JCS cells and mouse embryos

RT-PCR were performed on RNA samples from JCS cells incubated with midazolam (10 μg/ml) for 0, 1, 5, 18 and 48 hours, JCS cells treated with 50 μM biochanin A for 0, 1, 5, 18 and 46 hours, and mouse embryo at different developmental stages (7.5, 9.5, 11.5, 15.5 and 17.5 dpc). Two to three different cycle numbers ranging from 20-35 were performed. For some samples, touchdown PCR protocol was necessary to increase the specificity of primer annealing (Figure 4.7).

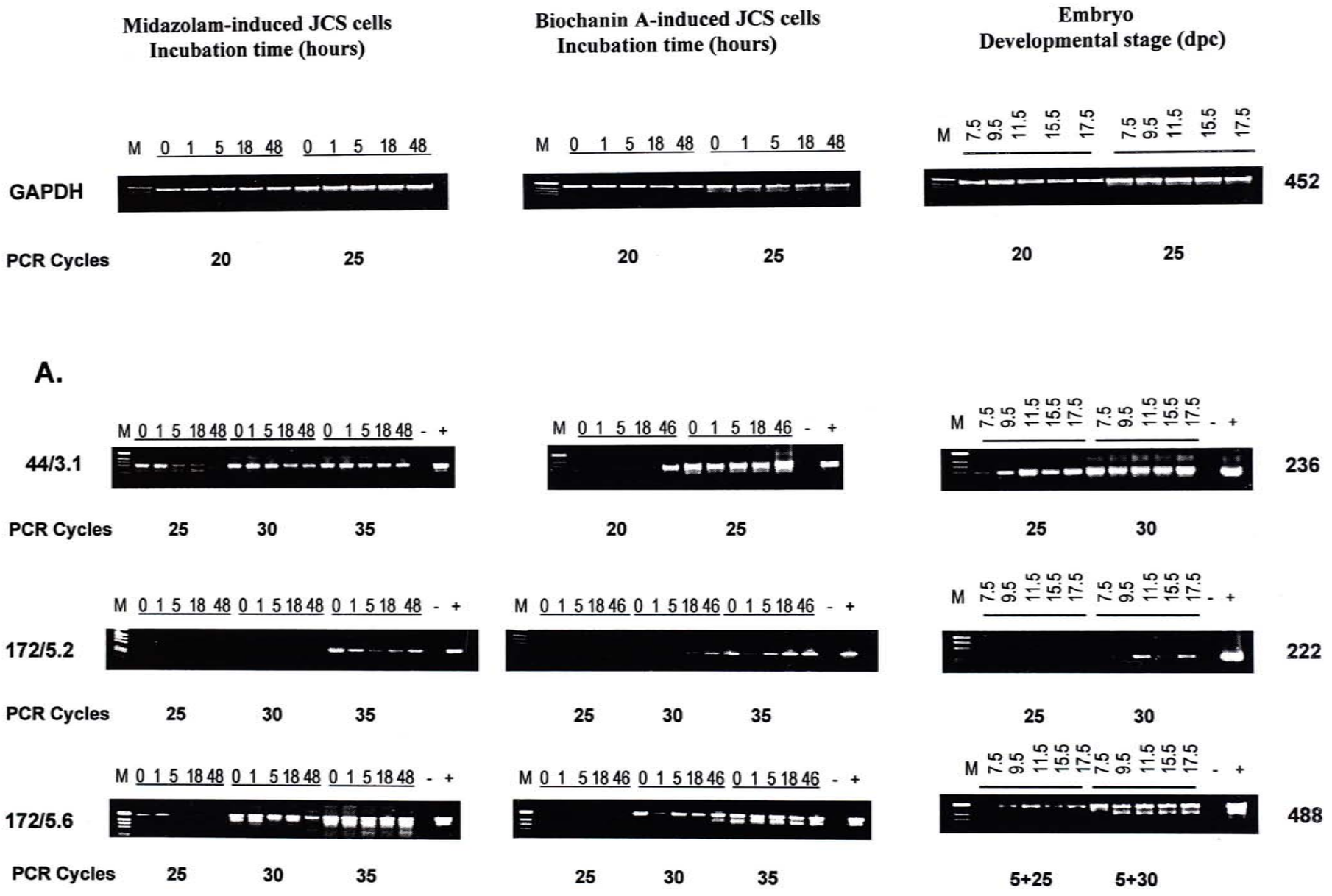
The expression profiles of the isolated genes (in the form of short fragments) during the midazolam-induced JCS cell differentiation can be divided into 6 types (Figure 4.7, column 1, Table 4.6). Expression patterns type A and B described genes that were down-regulated during the midazolam-induced differentiation. The expression patterns of three genes belonged to type A. They were down-regulated starting at the time point 5 hour (Figure 4.7 A). The expression patterns of other two genes belonged to type B where a more gradual decrease in expression was observed (Figure 4.7 B). Expression patterns type C and D described genes that were up-regulated after midazolam treatment. One gene having type C expression pattern was observed; it was transiently up-regulated at 1 hour after midazolam induction (Figure 4.7 C). On the other hand, two genes belonging to type D expression pattern showed a steady increase in expression level throughout the midazolam-induced differentiation (Figure 4.7 D). Finally, type E and F expression patterns indicated 'false positives' of this investigation. Two genes showed no change in expression level (type E) during the midazolam-induced differentiation (Figure 4.7 E). Two other genes belonging to type F expression pattern showed very low, if any, expression level as examined by 50 cycles of RT-PCR (Figure 4.7 F). Type A-D expression patterns were also found in the second cDNA probe screening in section 3.4.7 (Figure 3.9). Comparing the expression profile of these genes determined by RT-PCR and by the second round cDNA probe screening, we found that, of the 12 gene fragments studied by RT-PCR, the expression pattern of 7 gene fragments could be predicted in advance by analyzing the hybridization signals in the 2nd round screening (Table 4.6). However, there were a total of 8 genes (in terms of short fragments) which were

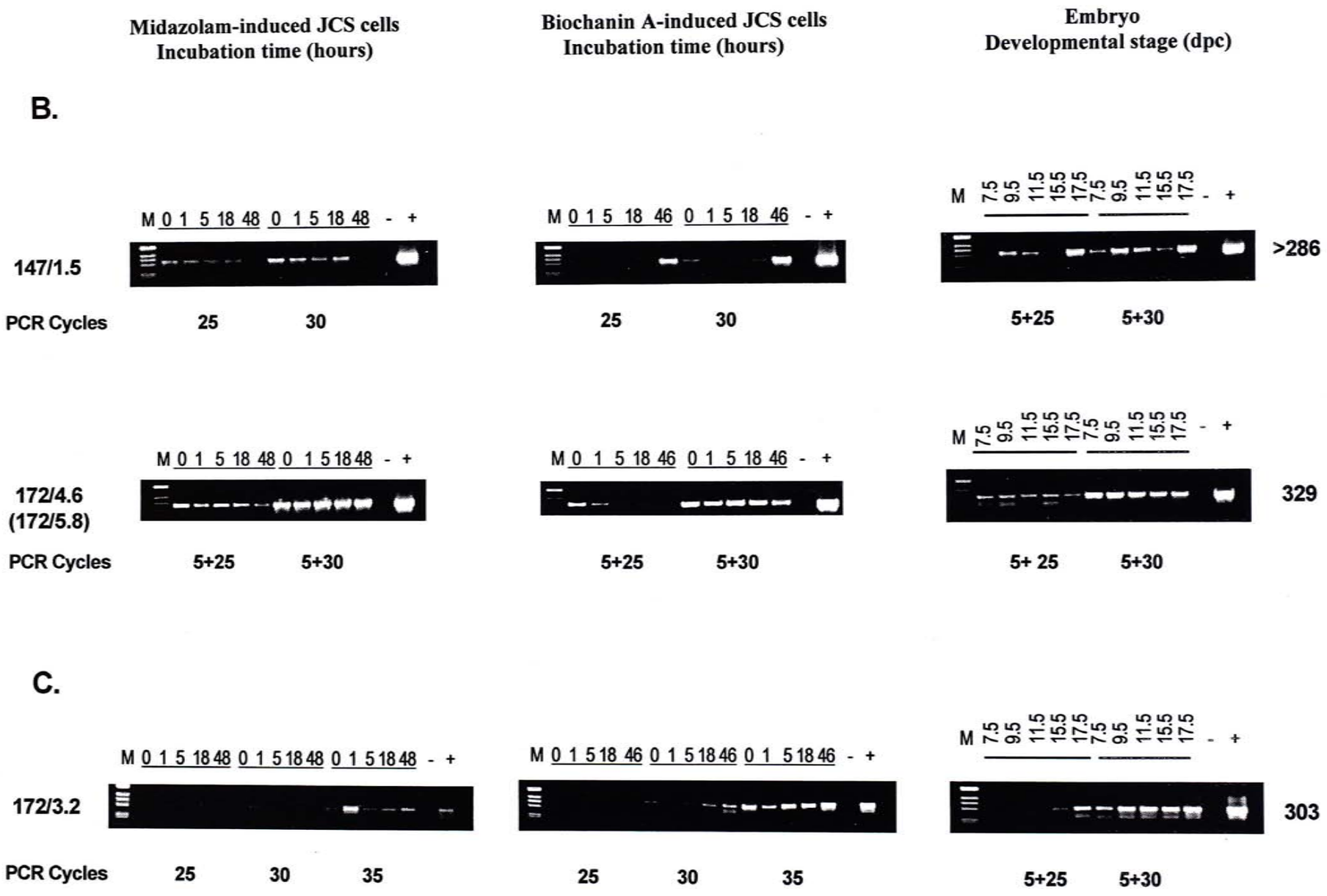
confirmed to be differentially expressed (i.e. genes that had expression patterns A-D) during midazolam-induced differentiation (Table 4.6). These isolated genes were then named according to their fragment numbers for the rest of the discussion.

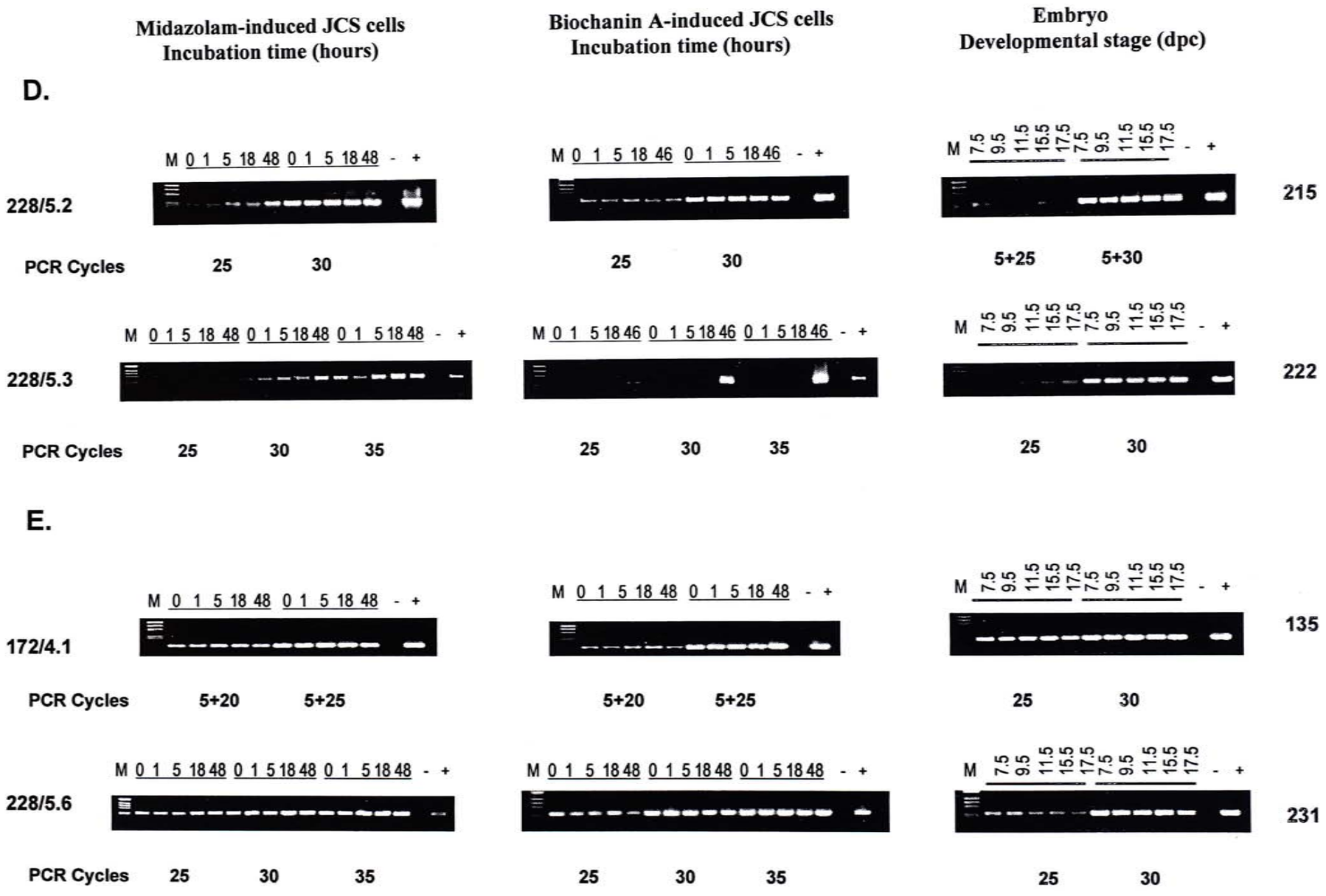
Table 4.6. **Expression profiles of the isolated gene (in the form of short fragments) in midazolam-induced JCS cells studied by RT-PCR and predicted by the second round cDNA probe screening in section 3.4.7**

Types of expression profile in midazolam-induced JCS cells	**Genes represented by the isolated fragments showing the type of expression profile determined by RT-PCR**	**Genes represented by the isolated fragments showing the type of expression profile in the 2nd round screening**
A. Constitutive expression at very early hour of midazolam treatment, down-regulation occurred at the time point 5 hour.	*44/3.1, *175/5.2, *172/5.6	*44/3.1, 172/3.2 (172/3.1), 172/4.6 (=172/5.8), 172/4.1, *172/5.2, *172/5.6
B. Late and gradual down-regulation	*147/1.5, 172/4.6 (172/5.8)	*147/1.5, 147/1.6
C. Transient up-regulation at early hour (at 1 hour)	*172/3.2	172/2.8, 172/3.8 (related to *172/3.2)
D. Steady up-regulation	*228/5.2, *228/5.3	147/1.4, *228/5.2, *228/5.3, 228/5.6
E. No change in expression as observed in 25 cycles of RT-PCR	172/4.1, 228/5.6	not applicable
F. No expression or very low expression as determined by 50 cycles of RT-PCR	147/1.4, 147/1.6	not applicable

* Gene fragments with the corresponding genes that showed the same expression pattern determined by both RT-PCR and predicted by the second round screening. The genes represented by 8 fragments (A-D) were confirmed to be differentially expressed in midazolam-treated JCS cells.


Midazolam-induced JCS cells
Incubation time (hours)
Biochanin A-induced JCS cells
Incubation time (hours)
Embryo
Developmental stage (dpc)
M 0 1 5 18 48 0 1 5 18 48
M 7.5 9.5 11.5 15.5 17.5 7.5 9.5 11.5 15.5 17.5
GAPDH
452
PCR Cycles
20
25
A.
44/3.1
M 0 1 5 18 46 0 1 5 18 46 - +
236
25
30
35
172/5.2
222
172/5.6
488
5+25
5+30


Midazolam-induced JCS cells
Incubation time (hours)
Biochanin A-induced JCS cells
Incubation time (hours)
Embryo
Developmental stage (dpc)
B.
M 0 1 5 18 48 0 1 5 18 48 - +
M 0 1 5 18 46 0 1 5 18 46 - +
M 7.5 9.5 11.5 15.5 17.5 7.5 9.5 11.5 15.5 17.5 - +
147/1.5
>286
PCR Cycles
25 30
25 30
5+25 5+30
M 0 1 5 18 48 0 1 5 18 48 - +
M 0 1 5 18 46 0 1 5 18 46 - +
M 7.5 9.5 11.5 15.5 17.5 7.5 9.5 11.5 15.5 17.5 - +
172/4.6
(172/5.8)
329
PCR Cycles
5+25 5+30
5+25 5+30
5+ 25 5+30
C.
M 0 1 5 18 48 0 1 5 18 48 0 1 5 18 48 - +
M 0 1 5 18 46 0 1 5 18 46 0 1 5 18 46 - +
M 7.5 9.5 11.5 15.5 17.5 7.5 9.5 11.5 15.5 17.5 - +
172/3.2
303
PCR Cycles
25 30 35
25 30 35
5+25 5+30


Midazolam-induced JCS cells
Incubation time (hours)
Biochanin A-induced JCS cells
Incubation time (hours)
Embryo
Developmental stage (dpc)
D.
M 0 1 5 18 48 0 1 5 18 48 - +
M 0 1 5 18 46 0 1 5 18 46 - +
M 7.5 9.5 11.5 15.5 17.5 7.5 9.5 11.5 15.5 17.5 - +
228/5.2
215
PCR Cycles
25 30
25 30
5+25 5+30
M 0 1 5 18 48 0 1 5 18 48 0 1 5 18 48 - +
M 0 1 5 18 46 0 1 5 18 46 0 1 5 18 46 - +
M 7.5 9.5 11.5 15.5 17.5 7.5 9.5 11.5 15.5 17.5 - +
228/5.3
222
PCR Cycles
25 30 35
25 30 35
25 30
E.
M 0 1 5 18 48 0 1 5 18 48 - +
M 0 1 5 18 48 0 1 5 18 48 - +
M 7.5 9.5 11.5 15.5 17.5 7.5 9.5 11.5 15.5 17.5 - +
172/4.1
135
PCR Cycles
5+20 5+25
5+20 5+25
25 30
M 0 1 5 18 48 0 1 5 18 48 0 1 5 18 48 - +
M 0 1 5 18 48 0 1 5 18 48 0 1 5 18 48 - +
M 7.5 9.5 11.5 15.5 17.5 7.5 9.5 11.5 15.5 17.5 - +
228/5.6
231
PCR Cycles
25 30 35
25 30 35
25 30

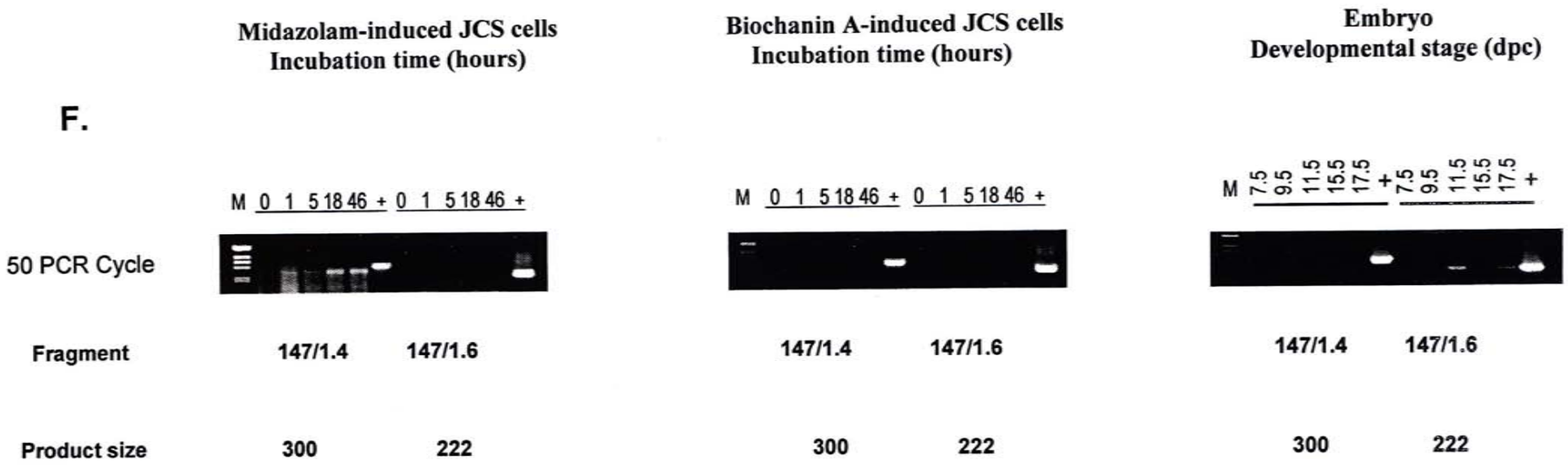


Figure 4.7. **Expression profiles of the cloned genes during JCS cell differentiation induced by midazolam or biochanin A and during embryonic development.** Total RNAs were obtained from either JCS cells induced by midazolam or biochanin A at different time points (0, 1, 5, 18, 46/48 hours) and embryos at different development stages (7.5, 9.5, 11.5, 15.5, 17.5 dpc). RT-PCR were performed on these total RNAs using appropriate primers (see table 4.5) at the PCR cycles indicated. For 44/3.1, upper and lower primer 1 were used. The expression profiles were grouped into 5 categories with respect to the expression patterns observed in midazolam-induced JCS cells. A. Constitutive expression but down-regulation at early hours. B. Constitutive expression but gradual down-regulation at late hours. C. Transient up-regulation at early hours. D. Steady up-regulation. E. No change in expression observed at 25 cycles. F. No expression / very low expression at 50 cycles. GAPDH amplified by specific primers (Table 2.1) was used to normalize each group of the samples under comparison. For PCR cycles, 5+N means 5 touchdown PCR cycles starting from (Tm-10) °C as annealing temperature at the first cycle which dropped by 1 °C per each consecutive cycle for a total of 5 followed by N cycles having an annealing temperature of (Tm-5) °C. The expected product size for each set of RT-PCR was shown on the right or as indicated. M=1Kb ladder. + : PCR using the same pairs of primers on recombinant plasmids harboring the corresponding fragments indicated as the leftmost column.

A. Genes down-regulated at 5 hours in midazolam-treated JCS cells

Genes 44/3.1, 172/5.2 and 172/5.6 showed a constitutive expression in untreated JCS cells and in cells treated with midazolam (10 μg/ml) for 1 hour. After 5 hour incubation with midazolam, the expression of the genes was decreased and remained at that low level thereafter (Figure 4.7 A, column 1, Table 4.7). On the other hand, the expression profiles of these genes in biochanin A-treated samples and at different stages of embryonic development were very different. The three genes also showed distinct expression profiles from one another in these samples (Figure 4.7 A, column 2 and 3, Table 4.7). In contrast to the expression profile of midazolam-treated samples, the gene 44/3.1 had a basal expression in untreated JCS cells and JCS cells treated with 50 μM biochanin A for 1, 5 and 18 hours. However, the expression level was up-regulated significantly after incubation with biochanin A for 46 hours. The expression pattern of genes 172/5.2 and 172/5.6 in biochanin A-treated samples were similar; both of them were transiently down-regulated at 1 hour after incubation with biochanin A. During embryonic development, genes 44/3.1 and 172/5.2 were more alike in their expression profile, where biphasic expression peaked at 11.5 and 17.5 dpc were observed. For gene 172/5.6, the expression level was up-regulated after 9.5 dpc and sustained at that elevated level throughout the rest of the development.

B. Genes steadily down-regulated in midazolam-treated JCS cells

The expression levels of two genes 147/1.5 and 172/4.6 (=172/5.8) were down-regulated by midazolam at late hours in a gradual manner (Figure 4.7 B, column 1, Table 4.7). The down-regulation in expression was notable at 48 hours for both genes. Despite their similar expression profiles in midazolam-treated cells, the expression patterns of the two genes were completely different in biochanin A-treated JCS cells and during embryonic development (Figure 4.7 B, column 2 and 3, Table 4.7). While the expression level of the gene 147/1.5 was down-regulated in midazolam-treated samples, its expression level was up-regulated significantly at 46 hours after incubation with biochanin A. In contrast, the gene 172/4.6 (=172/5.8) had a closer expression pattern in both types of samples; the expression level was down-

regulated in both midazolam- and biochanin A-treated samples. However, the change was more gradual and in a smaller degree in midazolam-treated JCS cells. During embryonic development, the expression of the gene 172/4.6 (=172/5.8) showed no change throughout the process. On the contrary, the expression of the gene 147/1.5 was particularly high at 9.5 and 17.5 dpc.

C. Gene transiently up-regulated at 1 hour in midazolam-treated JCS cells

The unique gene 172/3.2 was transiently up-regulated at 1 hour in midazolam-treated samples (Figure 4.7 C, column 1, Table 4.7) but transiently down-regulated at 1 hour in biochanin A-treated samples. It also showed a steady increase in expression level during embryonic development (Figure 4.7 C, column 2 and 3, Table 4.7).

D. Genes steadily up-regulated in midazolam-treated JCS cells

The expression levels of genes 228/5.2 and 228/5.3 were only slightly and steadily increased in midazolam-treated JCS cells (Figure 4.9 D, column 1, Table 4.7). The expression level of 228/5.2 was essentially constant in biochanin A-induced JCS cells and during embryonic development. The expression of the gene 228/5.3 was slightly increased during embryonic development and a remarkable increase in expression was observed in biochanin-A treated samples at 46 hours (Figure 4.7 D, column 2 and 3, Table 4.7).

E. Genes showing constitutive expression in midazolam-induced JCS cells

Genes 172/4.1 and 228/5.6 showed no change in expression level in midazolam-treated samples (Figure 4.7 E, column 1, Table 4.7) and in biochanin A-treated JCS cells (Figure 4.7 E, column 2, Table 4.7). The expression level of gene 172/4.1 was also unchanged during embryonic development but a very marginal decrease in expression was found for 228/5.6 during embryonic development (Figure 4.9 E, column 3, Table 4.7).

F. Genes showing no/low expression in midazolam-treated JCS cells

No or very low level of expression was found in midazolam-treated samples for genes 147/1.4 and 147/1.6 (Figure 4.7 F, column 1, Table 4.7) The same observation was also found in biochanin A-treated cells and during embryonic development. The expression of the gene 146/1.6 was barely detectable after 50 cycles of RT-PCR during embryonic development and an increased expression peaked at 11.5 dpc followed by a decrease in expression was observed (Figure 4.7 F, column 2 and 3, Table 4.7).

Table 4.7 : **Summary of expression profiles in different samples.**

Gene fragment (Matching sequence in the Genbank)	Midazolam-treated samples	Biochanin A-treated samples	Embryo at different developmental stages
A.			
44/3.1 (contains MT sequences)	Down-regulation at 5 hours	Up-regulation at 46 hours	Biphasic regulation peaked at 11.5 and 17.5 dpc
172/5.2	Down-regulation at 5 hours	Transient down-regulation at 1 hour	Biphasic regulation peaked at 11.5 and 17.5 dpc
172/5.6	Down-regulation at 5 hours	Transient down-regulation at 1 hour	Up-regulation sustained after 9.5 dpc
B.			
147/1.5	Steady down-regulation	Up-regulation at 46 hours	Biphasic regulation peaked at 9.5 and 17.5 dpc
172/4.6 (172/5.8)	Steady down-regulation	Prominent down-regulation after 1 hour	No change in expression
C.			
172/3.2	Transient up-regulation at 1 hour	Transient down-regulation at 1 hour	Up-regulation at 17.5 dpc
D.			
228/5.2 (Glucocerebrosidase mRNA)	Steady up-regulation	No change in expression	No change in expression
228/5.3 (S25 ribosomal protein mRNA)	Steady up-regulation	Up-regulation at 46 hours	Marginal steady up-regulation
E			
172/4.1	No change in expression	No change in expression	No change in expression
228/5.6 (Initiation factor)	No change in expression	No change in expression	Marginal down-regulation
F.			
147/1.4	No expression	No expression	No expression
147/1.6	No expression	No expression	Up-regulation peaked at 11.5 dpc

4.5 Discussion

4.5.1 Sequence analysis of isolated gene fragments

Sequence analysis is important in determining whether the genes are known or previously unknown. In addition, it provides information for designing specific primers for further expression pattern analysis.

The sizes of the subcloned fragments were in the range of about 300-900, comparable to other reports of RAP-PCR. On analysis, some of these subcloned fragments were related (Figure 4.4 A). The parent fragment 172/4 and 172/5 were adjacent bands on RAP-PCR gel (not shown). Thus, we suspected that their derived fragment 172/4.6 and 172/5.8 were the same contaminated species in the gel slices. In contrast, the relationship between 172/2.8, 172/3.1, 172/3.2 and 172/3.8 were more difficult to determine. Again, 172/2 and 172/3 were close fragments on the gel (not shown). However, their derived fragments were only identical by a stretch of short sequence (Figure 4.4 B). The longer sequence 172/2.8 or 172/3.8 was not detected on gel analysis of the re-amplified products (not shown) before subcloning. They contained additional arbitrary primer sequence other than the two flanking the fragment. From the location of the arbitrary primer sequence, these long fragments did not appear to be generated from ligation of two short fragments. These long fragments were possible artifacts generated during the subcloning procedures or by recombination events in the bacterial host. Since the four fragments 172/2.8, 172/3.1, 172/3.2 and 172/3.8 were related, only one of them 172/3.2 having proper arbitrary primer sequence on both ends was further analyzed.

In homology search, a few fragments matched with the known sequences in the Genbank. Fragment 44/3.1 contained a stretch of sequence (~359 bp) that matched the whole Mouse Transcript (MT) sequence while the rest of the fragment sequence being unique (Heinlein *et al.*, 1986). The MT family consist of short interspersed repetitive sequence (SINEs) with structural features of retroposons; a 400 bp consensus sequence was established (Bastien and Bourgaux, 1987). Of the 400 bp

consensus, 44/3.1 (part of its whole sequnece) matched very well along the whole sequence with 90-91 % identities (Figure 4.6, A). The alignment only interrupted at the 'gap' of undetermined sequences. This strongly suggested 44/3.1 is a member of the MT family. MT sequences have been found in the 5' region of rat cytochrome P450 oxidoreductase gene (Gonzalez and Kasper, 1983) and the mouse Ins sequences (Sylla *et al.,* 1984). The copy number of MT sequence within mouse genomic DNA was determined to be 4-9 X 10^4 per haploid genome. Although there is no study on the expression of MT sequence in hematopoietic tissues, the expression of the sequences was found to be transcribed preferentially in the brain in comparison to other tissues like kidney (Heinlein *et al.,* 1986). MT sequence was also found to be the most abundant repetitive sequence in the brain (Heinlein *et al.,* 1986). No hints on the function of the sequence or the corresponding gene can be deduced from the current findings. Thus, studying the expression profile of the gene 44/3.1 in midazolam-induced JCS cells may yield us insights into the role of the MT family genes and /or the particular member gene that fragment 44/3.1 represents.

Fragment 228/5.2 was homologous with glucocerebrosidase mRNA. The whole fragment aligned with the gene with 97% identities (Figure 4.5, C). As glucocerebroside is a glycolipid which may contribute to cell-cell interactions or signaling, regulation of glucocerebrosidase may affect the expression of these glycolipids and the response of JCS cells to external stimuli.

Two other fragments, 228/5.1 and 228/5.3 matched with 28S ribosomal RNA and S25 ribosomal protein mRNA sequences respectively. The whole fed-in sequence 228/5.1 matched with ribosomal 28S RNA with 94-95% identities (Figure 4.6, B). Since rRNA is expected to be more relevant to the functional aspects than to the differentiation program, we did not proceed further to analyze the gene. Fragment 228/5.3 had a good match with rat S25 ribosomal protein mRNA or its psuedogene (85-87% identities) (Figure 4.6 C). Finally, fragment 228/5.6 had high homology with the human initiation factor (94% identities) (Figure 4.8 D). The gene, together with 28S rRNA and S25 ribosomal protein, are the basic machinery of translation. The

direct relation of these gene with myeloid cell differentiation commitment was not seen.

The remaining fragments did not have significant matches with sequences in the database. However, it is still too early to conclude that all these fragments belonged to novel genes as the fragments may fall in the region outside the coding sequence. Sequences of untranslated region are not always be reported in the Genbank. Thus, characterized genes may not be detected during homology search using these RAP-PCR fragments. Hence, no conclusion can be made without first obtaining the full-length cDNA. Full-length cDNA can be obtained by conventional cloning method or by a more effective method 'rapid amplification of cDNA ends (RACE)' (Frohman *et al.,* 1988).

4.5.2 Expression profiles of the isolated genes

Gene specific primers were designed for the expression profile study by RT-PCR of each isolated gene.

Fragment 44/3.1 was suggested to be a member of the MT gene family (Table 4.4 and Figure 4.6 A). Although the exact gene has not been determined, down-regulation of the gene or genes that 44/3.1 represents correlated with the midazolam-induced monocytic/granulocytic differentiation (Figure 4.7, A). On the other hand, no down-regulation but rather an up-regulation of the gene was accompanied with biochanin A-induced monocytic differentiation after 46 hours. Since midazolam- and biochanin A-induced differentiation were different in lineage specification, the difference in expression pattern of the gene may be important in lineage commitment. Nevertheless, we cannot exclude the possibility that these changes are inducer-specific. During embryonic development, the gene was specially increased in expression from 7.5 to 11.5 dpc, slight decrease in expression at 15.5 dpc and increased again at 17.5 dpc. From this temporal expression pattern, the gene may be required particularly in organogenesis and late fetal growth. The gene may also be specially required in the development of hematopoietic tissue, as 11.5 dpc marked the time when fetal liver erythropoiesis, myelopoiesis and lymphopoiesis started. At 17.5 dpc, the major site of hematopoiesis has switched to the bone marrow (reviewed in Paulson and Bernstein, 1995) (Figure 4.7 A).

Like 44/3.1, down-regulation of the gene 172/5.2 was observed in midazolam-treated samples at the 5th hour. Transient down-regulation of the gene was also detected in biochanin A-treated JCS cells at 1 hour followed by a late increase in expression level. These suggested that down-regulation of the gene may be commonly required for both biochanin A- and midazolam-induced differentiation at early hours but a later increase in gene expression may only be required for biochanin A- but not midazolam-induced differentiation. Again, specifically higher expression level of the gene was found at 11.5 and 17.5 dpc during embryonic development. This suggested that the gene may be important in late organogeneisis (11.5 dpc) and late

fetal growth and development (17.5 dpc) and/or responsible for liver hematopoiesis (11.5 dpc) and bone marrow hematopoiesis (17.5 dpc) (Figure 4.7A).

172/5.6 was down-regulated during the midazolam-induced JCS cell differentiation. Similar to 172/5.2, this gene was only transiently down-regulated at 1 hour by biochanin A and up-regulated at a later time point. This may imply that down-regulation of the gene was not specific to one inducer and down-regulation of the gene was important for both types of differentiation. However, a later rebound in expression level was specific to biochanin A-induced differentiation. Unlike the expression shown by the two genes mentioned above, 172/5.6 was induced after 9.5 dpc during embryonic development. This suggested the gene may be involved in embryogenesis starting from early organogenesis (9.5 dpc) towards the end of the embryonic development (Figure 4.7 A).

147/1.5 was down-regulated slightly during the midazolam-induced differentiation. Conversely, the gene was up-regulated significantly after incubation with biochanin A for 46 hours. The degree of change (slight increase) in midazolam-induced samples and the expression pattern (late up-regulation) in biochanin A-treated samples did not suggest a contribution of the gene in the differentiation commitment. However, the gene may be responsible for some functional aspects of monocytes as the gene is specifically enhanced in the biochanin A-induced cells which were committed entirely towards the monocytic pathway. Interestingly, the gene was highly expressed at 9.5 dpc and 17.5 dpc in comparison to other developmental stages during embryogenesis. These time points coincided with organogenesis and hematopoietic precursor cell activities in AGM region (9.5 dpc) as well as late fetal growth and bone marrow hematopoiesis in the embryo (17.5 dpc) (Paulson and Bernstein, 1995) (Figure 4.7 B).

172/4.6 was down-regulated in differentiation initiated by midazolam. The same type of change was more obviously shown in differentiation caused by biochanin A under the same PCR cycle number amplification. This suggested the change in gene expression (down-regulation) was an event common to both typs of

differentiation. During embryonic development, the gene was constitutively expressed at different stages indicating that the gene was not developmentally regulated but required at all stages during embryogeneisis (Figure 4.7 B).

The gene 172/3.2 was transiently up-regulated at 1 hour in midazolam-treated JCS samples. Interestingly, transient down-regulation at 1 hour of the gene was observed in biochanin A-induced JCS samples. Such specific expression profiles reflected the different lineage preference of the cells triggered by different inducers. However, whether this 'opposite' expression pattern is deterministic in lineage choice needs further direct proof. In embryo, the gene was expressed at a higher level at 17.5 dpc but not in other stages suggesting that the gene may be more important for late development of embryo/fetus (Figure 4.7 C).

Fragment 228/5.2 has been aligned with glucocerebrosidase (mRNA sequence) (Figure 4.7 C) which was slightly enhanced in expression during midazolam-induced differentiation. On the other hand, the expression was unchanged in biochanin A-induced differentiation. Thus, the increased expression of glucocerebrosidase may be specific to the monocytic/granulocytic differentiation caused by midazolam or to the differentiation pathway initiated by midazolam. During embryonic development, the expression of the gene was roughly the same (Figure 4.7 D).

Fragments 228/5.3 and 228/5.6 were matched with ribosomal protein S25 mRNA (and its psuedogene) and initiation factor sequence respectively (Figure 4.6 B and C). Although the expression of the gene 228/5.3 was increased slightly in the midazolam-treated samples and a more obvious increase in expression was found in biochanin A-treated sample at 46 hour, we do not expect such changes be relevant to the differentiation commitment (Figure 4.7 D). For gene 228/5.6 (initiation factor gene), the expression was unaltered in both midazolam- and biochanin A-induced JCS cell differentiation (Figure 4.7 E). Taken together, the translation machinery may be enhanced during the differentiation through the increase in the S25 ribosomal proteins. Also, the isolated fragment 228/5.6 was a false positive of this investigation. The gene it represents was predicted to be up-regulated during midazolam-induced

differentiation in the second cDNA probe screening but this expression pattern was shown 'not true' by RT-PCR (Table 4.6). In embryo, the change in expression of these two genes was also negligible at different stages confirming the 'house-keeping' nature of these genes.

Gene 172/4.1 was found constitutively expressed during the midazolam- and biochanin A-induced differentiation. This result was opposed to the prediction in the second screening (false positive). Continued expression of the gene was also found throughout the embryonic development (Figure 4.7 E).

The expression of gene 147/1.4 was not detected by 50 cycles of RT-PCR during midazolam-induced or biochanin A-induced JCS cells. No expression of the gene was found during embryonic development as well. The expression of 147/1.6 was undetectable in midazolam-induced JCS cells but a barely detectable expression was found at 46 hours of biochanin A-treated sample. Despite the low expression level of 147/1.6, the expression was peaked at 11.5 dpc during embryonic development. This suggested a possible role of the gene during organogenesis in embryonic development (Figure 4.7 F).

In conclusion, 8 out of the 12 gene fragments (omitting the identical and related fragments) selected after the second round screening were confirmed to be differentially expressed genes during midazolam-induced cell differentiation. This statistics was comparable to, if not better than other reports using similar methods. For example, in Liang *et al.* (1993), 5 out of 15 fragments were confirmed to be differentially expressed, 4 fragments were false positives and 6 failed to show expression as examined by northern blot. Besides, in this report, all of the fragments except 147/1.4 representing genes that were expressed in normal tissues (embryo) as well as in leukemia cells. Since little can be concluded from the present studies on the nature and function of the isolated gene fragment in both myeloid differentiation and in embryogenesis, further work must be carried out. In particular, whether the corresponding genes of the isolated gene fragments with differential expression are essential to the differentiation of JCS cells is our main concern. Further work can also

be done to see if the genes can initiate JCS cell differentiation by over-expressing the genes. Also, investigation can be carried out to elucidate those genes that showed differential expression during embryonic development. The genes may be involved in the development of hematopoietic tissue specifically and/or in non-hematopoietic tissues in general.

Chapter Five General Discussion

5.1 Studies on leukemic cell differentiation

5.1.1 Differentiation pathways revealed by different inducers

On the way to delineate the underlying mechanism of hematopoiesis and its control, we started by studying the induced cell differentiation of a newly-developed myeloid leukemia cell model, WEHI-3B JCS (Mak *et al.,* 1993). Like many other well-characterized models, this cell line could be induced to differentiate by a repertoire of defined inducers such as PKC activator (phorbol 12-myristate 13-acetate, PMA), bacterial product (lipopolysaccharide, LPS) and growth factors (IL-1α, IL-1β, TNF-α, TNF-α + IL-4, TNF-α+ IFN-γ) (Chan *et al.,* 1995; Leung *et al.,* 1994; Mak *et al.,* 1993, 1994). Only recently, biochanin A and midazolam were also shown to be potent inducers of JCS cells (personal communication with Dr, N.K. Mak).

Despite the use of these different inducers, JCS cells may have followed the same differentiation pathway. It was found that endogenous TNF-α mRNA production was triggered during both the TNF-α- and PMA-induced JCS cell differentiation. Neutralizing antibodies against TNF-α could abrogate both of these induced differentiation (Mak *et al.,* 1993; Chan *et al.,* 1995). Besides, pertussis toxin (a modulator of G protein) had enhanced effect on TNF-α– as well as LPS- and PGE_2-stimulated differentiation (Mak *et al.,* 1994, authors' unpublished data). These findings suggested that some common steps in differentiation are triggered by different inducers. Similarly, the same immediate early response genes and tyrosine phosphorylation were triggered by both IL-6 and LIF during M1 cell differentiation (Lord *et al.,* 1991).

In contrast to the above observations, different inducers have been shown to mediate differentiation by distinct pathways. For instance, both IFN-γ and vitamin D_3

were able to induce differentiation of THP-1 cells. However, IFN-γ but not vitamin D_3 induced cytokine (IL-1β, TNF-α and IL-6) production (Vey *et al.,* 1992). Also, the differentiation-inducing agents PMA and retinoic acid of THP-1 cells had different effects on the *src* family tyrosine kinase genes and c-*myb* expression. PMA but not retinoic acid mediated THP-1 cell differentiation through AP-1 (Matikainen and Hurme, 1994). In TNF-α-induced JCS cell differentiation, the expression levels of IL-1α, IL-1β, TNF-α and IFN-γ were enhanced (Chan *et al.,* 1995) at 18 hours. In the current study, however, JCS cells triggered either by biochanin A or midazolam failed to increase the production of IL-1α, IL-1β nor TNF-α significantly. While IL-1α mRNA level was increased at 46 hours in biochanin A-treated cells, there was no detectable level of IL-1α in midazolam-treated cells. The expression of IL-1β was increased at 46 hours after incubation with biochanin A but reduced after incubation with midazolam in JCS cells. TNF-α gene expression was also slightly increased in JCS cells when triggered by biochanin A but remained unchanged when induced by midazolam. Taken together, TNF-α, biochanin A and midazolam have stimulated unique pathways for JCS cell differentiation.

Autocrine production of IL-1β and TNF-α and their effects on myeloid cell differentiation have been shown not only in leukemia cells (Chan *et al.,* 1995; Magrinat *et al.,* 1992) but also in normal hematopoietic progenitors (Witsell and Schook, 1992; Watari *et al.,* 1996). This suggested that the endogenously produced cytokines may be involved in the regulation of normal hematopoiesis. Since biochanin A and midazolam appeared to have bypassed the differentiation pathway mediated by endogenous TNF-α and IL-1β, other differentiation pathway may exist. Hence, to extend our study on cytokine involvement in myeloid cell differentiation, the gene expression of LIF, LIF-R and IL-4 in JCS cells were studied. IL-4, LIF-R (both membrane and soluble forms) showed no expression in either biochanin A- or midazolam-induced JCS cells whereas the expression of LIF was up-regulated by biochanin A and unaltered by midazolam. Accordingly, the autocrine effect of LIF and IL-4 on JCS cell differentiation triggered by biochanin A and midazolam can be ruled out.

5.1.2 Lineage preference during differentiation

JCS cells have been demonstrated to differentiate into either monocytes or granulocytes (Leung *et al.*, 1994; Mak *et al.*, 1993, 1994, 1996). So far, all the defined inducers reported (Leung *et al.*, 1994; Mak *et al.*, 1993, 1994) and biochanin A (personal communication with Dr. N. K. Mak) led the cells towards the monocytic lineage. In contrast, midazolam was able to drive JCS cells to produce both granulocytes and monocytes (personal communication with Dr. N. K. Mak). A balance between exogenous cytokines have been suggested to bias the lineage choice (monocytic or granulocytic) of myeloid progenitor (Snoeck *et al.*, 1996). Thus, it is possible for JCS cells to follow different lineage pathway through the autocrine action of endogenously produced cytokine. Comparing the cytokine and cytokine receptor gene expressions of biochanin A-induced (monocytic) and midazolam-induced (granulocytic and monocytic) differentiation may provide us insight into lineage commitment.

Most of the cytokine and cytokine receptor gene expression studied were changed differently during the induced differentiation of JCS cells having different lineage preference (Table 2.3). Up-regulation of IL-1β and IL-3 expression levels were found in the biochanin A-induced JCS cells but down-regulation of their expression levels were observed in midazolam-induced cells. LIF and IL-3Rα gene expression was increased during the differentiation of biochanin A-induced cells but unchanged during the differentiation of midazolam-induced cells. IL-1RtI and IL-3Rβ were unchanged in expression levels in JCS cells after incubation with biochanin A while increase in expression levels was observed after incubation with midazolam. The gene expression of IL-1α was induced during the differentiation of biochanin A-treated cells but not during the differentiation of midazolam-treated cells. Finally, the expression pattern of IL-1RtII remained unchanged after incubation with biochanin A but transiently decreased at 18 hours after incubation with midazolam. These results revealed that the cytokine and cytokine receptor mRNA phenotypes were very different between cells under two different treatments.

Nevertheless, these cytokines may not be responsible for lineage commitment if we consider also the degree of change in expression level and the time point at which the change occurred. The change of cytokine mRNA level at late time point (46 or 48 hours) might be associated with cell maturation and function. For example, IL-1 and LIF are readily produced by monocytic cells. The expressions of these genes were induced and increased only after 46 hour incubation with biochanin A, coincided with the time when the cells started to exhibit their maturing phenotypes. IL-1 has been reported to mobilize neutrophils. In the current investigation, the mRNA level of IL-1R (type I) was increased significantly in midazolam-induced JCS cells consisting of a considerable percentage of granulocytes (neutrophils). Hence, the enhanced level of IL-1R may be related to the response of the cells to IL-1 during the recruitment of mature neutrophils to the inflammatory site. Lastly, it is important to be reminded that these changes in gene expression pattern may simply be inducer-specific changes unrelated to either the differentiation event or cell function.

5.2 Differentiation program triggered by midazolam

5.2.1 Signaling pathways initiated by biochanin A and midazolam

Little is known about how biochanin A and midazolam initiate their signals for differentiation. It was demonstrated in this report that several important cytokines (by autocrine production) for myeloid cell as well as JCS cell differentiation were not involved.

Biochanin A was suggested to be an inhibitor of tyrosine protein phosphorylation (Besterman and Schultz, 1990). Protein tyrosine kinases have been implicated in the regulation of cell transformation and cell growth. Their activities are probably the earliest biochemical change that can be measured after ligand-receptor binding. The enzymes are usually responsible for phosphorylation of specific tyrosine residues in the cytoplasmic domains of receptors or receptor-associated signal-transducing subunits. This leads to the clustering of SH2-containing protein at the membrane. The tyrosine kinases (receptor-associated) can further phosphorylate the tyrosine residues on these SH2-containing proteins and activate them. A cascade of changes involving cytoplasmic calcium and protein kinase C or further SH2-containing proteins, GTP-binding protein and serine/threonine kinase may be initiated. Finally, transcription of the responder genes by transcriptional factors like AP-1, c-*myc* is triggered. By inhibiting the required tyrosine kinase for excessive proliferation signal or suppressing the over-expressing tyrosine kinase in leukemia cells may somehow restore the differentiation capacity of JCS cells.

Although the binding of midazolam onto the peripheral benzodiazepine receptors (PBR) has not been demonstrated, the anti-proliferative (Wang *et al.,* 1984a) and differentiation-inducing effects (Ishiguro *et al.,* 1987) through high and low affinity PBRs respectively have been proposed. Peripheral active benzodiazepines were also able to super-induce specifically c-*fos* that associated with the differentiation process (Curran and Morgan, 1985). PBRs are widely distributed in

monocytic cells and increase in expression upon maturation (Canat *et al.* 1993); it follows that midazolam may effect its differentiation-inducing action through PBRs. The important component of PBRs was suggested to be associated with a voltage dependent anion channel and an adenine nucleotide carrier but the effector system of the receptors has not been established (Parola *et al.,* 1993). Interestingly, a distant relative of biochanin A, chrysin (5,7-dihydroxyflavone) has been shown to bind to the central benzodiazepine receptor (CBR) (Wolfman *et al.,* 1994). Although CBR and PBR (section 1.3.3) are entirely different receptors, some ligands like diazepam do bind to both receptors. As a result, we could not exclude the possibilities that both midazolam and flavonoids like biochanin A accomplish their biological effects through PBRs.

All of these, however, are only speculation of how biochanin A and midazolam act in differentiation. It is still a long way to dissect the exact effector system initiated by the two molecules.

5.2.2 Differentially expressed genes during midazolam-induced differentiation

To search for genes that are central to the genetic program for JCS cell differentiation, we started by seeking genes that showed differential expression during the differentiation of JCS cells. In order to gather more information, we employed materials (RNA) from JCS cells induced by midazolam as it drove the cells not only to monocytic but also to granulocytic differentiation. We examined the gene expression of the differentiating cells at 0, 1, 5, 18 and 48 hours after incubation with midazolam. This time course study should encompass the expressions of immediate early response genes (e.g. c-*fos,* c-*myc*) to early response genes (e.g. c-*myb*) (1, 5 hours) and early to late response genes (e.g. some surface markers) (18, 48 hours).

To facilitate a time course study, we employed a modified protocol of RNA fingerprinting by arbitrarily-primed polymerase chain reaction (RAP-PCR), instead of using conventional methods like differential screening or subtractive hybridization. The current modified method is suited for a random search of a pool of genes that are differentially expressed among different samples. The problem of non-reproducibility and false positives of the original protocol (Welsh *et al.,* 1992) were greatly alleviated by employing two rounds of cDNA probe screening in this new protocol. 142 differentially amplified fragments were obtained initially from the fingerprints. At the end of the second round screening, only 12 subcloned fragments of distinct sequences remained. Among these 12 fragments, 8 fragments represented differentially expressed genes confirmed by reverse-transcription polymerase chain reaction (RT-PCR) (Figure 4.6 A-D). Only three out of these 8 fragments aligned with known sequences in the Genbank. These known sequences were MT sequence repetitive element DNA (44/3.1), mRNA for glucocerebrosidase (228/5.2), and ribosomal protein S25 (228/5.3).

5.2.3 Expression patterns of the isolated differentially expressed genes in midazolam- and biochanin A-induced JCS cells

A gradual increase in expression of glucocerebrosidase (228/5.2) was observed in midazolam-induced JCS cells and suggested an increased requirement for glycolipids (Figure 4.9 D). As a result, cell-cell interaction or other cell functions of JCS cells may be affected after incubation with midazolam. However, the expression level of this gene remained the same in JCS cells incubated with biochanin A suggesting no involvement of this gene in biochanin A-mediated differentiation. The expression of ribosomal protein (228/5.3) gradually increased during midazolam-mediated differentiation (Figure 4.9 D) but was increased more rapidly during biochanin A-mediated differentiation. This indicated that the translation machinery is generally enhanced to meet the demand during differentiation. Genes having MT sequences were found preferentially transcribed in the brain (Heinlein *et al.*, 1986) but the expressions in the hematopoietic tissue have not been studied. In this report, a gene belonging to the MT sequence family (44/3.1) was down-regulated significantly in JCS cells at 5 hours after midazolam incubation (Figure 4.9 A). This indicated that the gene may be specifically involved in the midazolam-induced or monocytic/granulocytic cell differentiation. The view is further supported by the fact that the gene expression was unaltered by biochanin A at early hours but only increased after incubation with biochanin A for 46 hours. Since down-regulation of the gene was associated with the differentiation event, this gene could be a negative regulator for midazolam-induced differentiation.

Besides these known genes, two of the unknown genes (172/5.2 and 172/5.6) showed marked down-regulation of expression at 5 hours after midazolam treatment. Interestingly, these two genes were only transiently down-regulated at 1 hour when treated with biochanin A (Figure 4.9 A). The expressions of two other unknown genes 147/1.5 and 172/4.6 were decreased gradually during the midazolam-induced differentiation. On the other hand, gene 147/1.5 was increased markedly in mRNA level at 46 hours while gene 172/4.6 was down-regulated sharply in JCS cells after incubation with biochanin A (Figure 4.9 B). Moreover, 172/3.2 was transiently up-regulated at 1 hour after induction by midazolam while a transient down-regulation of

the expression level at 1 hour was observed in biochanin A-treated cells (Figure 4.9, C).

Most of the genes found differentially expressed in midazolam-induced JCS cells have quite a different pattern of expression in JCS cells induced by biochanin A. This observation is consistent with the cytokine gene expression studied earlier. As stated before, biochanin A and midazolam-treated cells have different lineage preference during differentiation. By comparing the gene expression pattern in the two systems, we may be able to locate genes that are specific to a certain lineage. It is not uncommon to find genes that have different effects on different cell lineages. One of the notable examples was the zinc finger protein *egr-1*. While *egr-1* was found up-regulated during monocytic differentiation, no induction of expression was found during granulocytic differentiation. Also, *egr-1* is essential for monocytic differentiation and suppresses granulocytic differentiation (Nguyen *et al.,* 1993). Thus, it is likely that genes isolated in this study, having different expression patterns in biochanin A and midazolam-induced JCS cells, would have different effects on lineage commitment to monocytic or monocytic/granulocytic differentiation. However, all these changes may also be inducer-specific changes irrelevant to cell differentiation. Thus, careful interpretation and further investigation are necessary.

5.2.4 Myeloid genes in embryonic development

A number of genes involved in or essential to myeloid cell differentiation are also important in embryonic development. These genes code for either growth factors and their receptors or transcription factors (reviewed in Hogan *et al.,* 1994; Paulson and Bernstein, 1995; Simeone *et al.,* 1995). In particular, c-*myb* which was down-regulated upon myeloid cell differentiation also showed a distinct expression pattern during embryonic development. In embryo, the expression of c-*myb* was very low before 10.5 dpc. The expression was detected from 10.5 dpc and persisted throughout the rest of the developmental stage. *In situ* hybridization revealed that the gene was expressed not only in hematopoietic organs but also in non-hematopoietic tissues (Sitzmann *et al.,* 1995). This highly suggested that genes which were differentially expressed during midazolam-induced differentiation may also played a role in embryonic development. A study of the expression pattern of our newly identified genes during embryonic development should give us insight into the functions of the genes in both hematopoietic and non-hematopoietic systems.

In this study, some isolated genes that are differentially expressed during the JCS cell differentiation triggered by midazolam (and biochanin A) also appeared to be developmentally regulated as examined from the expression pattern (Table 4.7). For example, genes 44/3.1 (MT family sequence) and 172/5.2, with distinct patterns of expression in midazolam- and biochanin-A-induced cells, had enhanced expression particularly at 11.5 dpc and 17.5 dpc (Figure 4.9 A). These time points 11.5 dpc and 17.5 dpc coincided with the time of organogenesis (and the beginning of fetal liver hematopoiesis) and late fetal growth (and the beginning of adult-type hematopoiesis in fetus) in mouse development respectively. Similarly, gene 147/1.5 had expression peaked at 9.5 and 17.5 dpc (Figure 4.9 B). Thus, it is likely that the gene played a role in early organogenesis (and *de novo* hematopoiesis in AGM region), late fetal growth (and fetal hematopoiesis).

On the other hand, gene 172/5.6 was expressed at high level during the later stage of development (9.5-17.5 dpc) suggesting a general requirement in late growth

and development of the embryo (Figure 4.9 A). The expression level of the gene 172/3.2 was significantly higher at 17.5 dpc indicating that the gene may be specially required in fetal growth.

Whether the changes in gene expression levels described above are specifically involved in (or essential to) myelopoeisis in embryos or are also associated with the development of other tissues cannot be concluded from these results. Perhaps to solve this problem, it is a good idea to perform *in situ* hybridization on embryos at different developmental stages. c-*myb,* a myeloid gene, was located not only in the hematopoietic organs like fetal liver and thymus in embryo but also in the neural retina and epithelia of the respiratory tract as studied by *in situ* hybridization technique (Sitzmann *et al.,* 1995). In contrast, c-*jun* which initiates differentiation of monocytic cells (Li e*t al.,* 1994c) may play no part in fetal hematopoiesis. Its expression was limited to cells in the developing cartilage, gut and central nervous system in the embryos as examined by *in situ* hybridization (Wilkinson *et al.,* 1989). Although further speculation on function of our isolated genes in embryonic development and their relation with myeloid cell differentiation were limited, this study nevertheless provides some basic information about the isolated genes. This will be particularly useful in future characterization of the isolated genes which have not been reported previously.

5.3 Future studies of the isolated fragments

There are many approaches that can be used for isolating genes that are important in myeloid cell differentiation. Among the possibilities, we have chosen to correlate the expression profiles of a number of randomly selected genes with the differentiation event. From these differentially expressed genes, those that are important and essential for myeloid cell differentiation will be identified. However, because of the limited scope and time of this study, the project have to be stopped after the isolation of a couple of differentially expressed genes. However, the studies would not be meaningful until we have directly proved that the genes are also essential to or involved in myeloid cell differentiation.

Starting from the subcloned fragments obtained in this study, it may be beneficial to obtain the full-length cDNA to which the fragment belonged. Since the subcloned fragments may represent sequences of the non-coding region, the coding sequences must be known for further investigation. To prove whether an isolated gene is essential for JCS or myeloid cell differentiation, gene function studies are inevitable. Direct strategy by micro-injection of antibody against the target gene product may be one of the choices. Useful information about the functions of various oncogenes have been obtained (reviewed in Kovary, 1995). Increasingly important strategies like inducible expression system (Labow, 1995) and antisense techniques (Robinson-Benion and Holt, 1995) may still be better choices as our starting materials are the DNA sequences.

Inducible expression system (e.g. *lac* activator protein system) allowed precise control of the exogenous gene expression at a particular time. There are several isolated gene fragments (44/3.1, 172/5.2, 172/5.6) in this report that are down-regulated at 5 hours in JCS cells after midazolam incubation. Some important information of the genes may be revealed if we extend the expression of these genes beyond the 5 hour point. That is, if down-regulation of these genes are essential for JCS cell differentiation, the cells will continue to proliferate without differentiation even in the presence of midazolam. Alternatively, if the down-regulation are

important for lineage commitment, the cell may differentiate but with a different lineage preference. On the other hand, if the cells differentiate without any change under the enforced expression of these genes, there could be two interpretation : (1) The genes are not essential to the differentiation and lineage selection program of JCS cells. (2) The genes need other co-operative genes for function.

Alternatively, antisense techniques can be used to suppress the expression of these genes in untreated JCS cells. If the cells differentiate or alter their lineage choices upon inhibition of the gene by the antisense construct, the gene is probably very important negative regulator and sufficient factor for JCS cell differentiation or lineage development. On the contrary, if the cells does not differentiate upon antisense treatment or change in lineage specificity, the gene probably is not as crucial as predicted.

For up-regulated genes like 172/3.2 during the differentiation of JCS cells initiated by midazolam, antisense construct (preferentially with inducible regulation) can be used to see if the differentiation can be blocked or lineage choice can be altered in the presence of midazolam. Again, controlled expression of the gene in untreated JCS cells can be used to check if the gene is sufficient to initiate the differentiation program of JCS cells.

Instead of using ectopic expression or antisense techniques, gene targeting and knock-out techniques can be adopted in JCS cells, hematopoietic stem cells or in a whole animal for gene function study. The technique involves an introduction of a genomic DNA fragment of the desired gene or a non-functional gene which will then locate and recombine with the endogenous homologous sequences. The effect of excessive expression of a gene or no expression of a gene can be investigated accordingly.

As a recapitulation, it is absolutely important to prove the role of the isolated genes in this study by some direct strategies . Although the function of many genes (Table 1.1) can be predicted correctly by their expression patterns, there are far more

unsuccessful examples. For instance, the role of some protein kinases like *src, hck* and *fgr* in myeloid cell differentiation as predicted from their expression pattern was misleading. Both the mRNA and protein product of the c-*fgr* were shown to increase in expression during the induced differentiation of myeloid leukemia cells (U937, HL-60) and normal myeloblasts (Willman *et al.,* 1991). In addition, transfected c-*fgr* was also able to induce monocyte-specific enzyme in heterologous cells. However, it was found that homozygous knockout mice for c-*fgr* has no functional defect. Similar observations were also shown for c-*src* and c-*hck* (Varmus and Lowell, 1994). In other cases, genes that showed distinct pattern of expression during differentiation were finally proved to be involved in apoptosis, anti-proliferation or maturation. For example, the expression of c-*fes* (cytoplasmic tyrosine kinase) was increased upon differentiation of cells into monocytes or granulocytes. However, antisense techniques revealed that the gene has a function on programmed cell death (Manfredini *et al.,* 1993).

Assays for gene function are not without problems. First, members of the same gene family may compensate each other in functions. That is, the function of one gene abrogated by either anti-sense or knock-out techniques can be restored by some other members of the gene family. To suppress all the genes of the same kind may not be at all possible, partly because we may not be able to locate all the genes with redundant functions. Also, blocking all these genes may be lethal to the cell or the animal.

Second, it appears that myeloid differentiation is not entirely dependent on one master gene (transcription factor). Combination of transcription factors (master genes) may be required for the expression of other differentiation-related genes (responder genes). For example, c-*myb* and *C/EBPβ* are both required to induce the myeloid-specific gene *mim-1* (Ness *et al.,* 1993). Thus, if we study each gene in isolation, the crucial action of these genes may not be readily seen. Such problem may be able to be circumvented by studying functions of genes in combination. For example, where down-regulation of genes are associated with the differentiation event, like in the case of c-*myc* and *max* in M1 cells, their gene expression could be inhibited concomitantly by antisense technique. Such study clearly demonstrated that

terminal differentiation of the cells are only possible when expression of both but not either gene are blocked (Nguyen *et al.,* 1995). Hence, studying functions of genes in combinations may be more fruitful, especially for genes that may have interactions with one another (e.g. dimerization and competition for binding site).

5.4 Conclusion

In this report, we have found that biochanin A and midazolam mediated distinctive differentiation pathways of WEHI-3B JCS cells. The pathways do not involve the action of endogenously produced IL-1, TNF-α, IL-4 nor LIF. In addition, eight genes were isolated and confirmed to be differentially expressed during the midazolam-induced granulocytic and monocytic differentiation. Five of them were down-regulated and three of them were up-regulated during the midazolam-induced differentiation. All of these genes also showed different expression patterns in biochanin A-induced cells, suggesting different roles of the genes in the biochanin-induced monocytic differentiation. Besides the roles in myeloid differentiation, the genes may also be involved in embryonic development. To confirm the exact role of these isolated genes in myeloid cell differentiation, either acting alone or in combination with other genes, gene functional studies are suggested.

Hopefully, the results obtained in this project and in the follow-up studies, when compared with that from other myeloid cell models (both normal and leukemic) and *in vivo* studies, can lead to a greater appreciation of the hematopoietic system. This will in turn influence the concepts of leukemogenesis and perhaps, novel treatments based on restoring normal differentiation and maturation are possible in the near future.

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Appendix

A1. Ambiguity codes for sequencing

Code	Unresolved Bases
M	AC
R	AG
W	AT
S	CG
Y	CT
K	GT
V	ACG
H	ACT
D	AGT
B	CGT
N	ACGT

A2. Myeloid cell lines

Cell line	Origin	Brief description
32Dcl3	Bone-marrow derived myelomonoblastic murine cell line	Murine myeloid cell, generation time of 18 hours, IL-3 dependent, differentiates into granulocyte (6-14 days) upon stimuation with G-CSF in the absence of IL-3
HEL	Human, erythroleukemia	Induced to differentiate along erythroid or monocytic lineages
HL-60	Human promyelocytic leukemia	Expresses surface receptors for Fc fragment and complement, differentiates into monocytes triggered by PMAor Vitamin D_3 and into granulocytes stimulated by GM-CSF, DMSO, or RA, induced to produce TNF-α, activation of c-*ras* by point mutation and amplified expression of c-*myc*
K562	Human, blast crisis of chronic myeloid leukemia (progenitor stage)	Bipotent, induced to differentiate towards erythroid lineage by ara-C and myelomonocytic lineage by TPA
KG-1	Human, derived from patient with erythroleukemia, myeloblast-like	Consists of a predominant myeloblasts plus other cell types, induced to differentiate into monocyte by PMA
KG-1a	Derived from KG-1, at a stage more immature than its parental line	Unable to differentiate upon induction of PMA
M1 (D+)	Mouse (SL-strain) myeloblastic leukemia	Full differentiation into monocytes induced by IL-6 and LIF, partial differentiation by IL-1 or LPS and anti-proliferation by IFN-β and IFN-γ, monocytic differentiation upon vitamin D_3 induction
ML-1	Human myeloid cell line	Monocytic differentiation upon TPA induction
THP-1	Human monoblastic cells	Readily induced by PMA, vitamin D_3, and IFN-γ to produce monocytes
WEHI-3B(D+)	Murine myelomonocytic leukemia	Macrophage-like, secrete IL-3, induced by G-CSF to follow monocytic lineage, unresponsive to LIF
WEHI-3B(JCS)	Murine Balb/c, dervied from WEHI-3B (D^-)	Induced by TNF-α, IL-1, LPS, PMA and biochanin A to produce monocytes but induced by midazolam to produce cells of monocytic and granulocytic lineages
U937	Human monoblastic leukemia, promonocytic, histiocytic	PMA, 1-2,5-dihydroxyvitamin D_3 induced monocytic differentiation, more determined than HL-60

A3. Details of manufacturer's products

1. DIG oligonucleotide 3'end labelling kit **Boehringer Mannheim 1362372**

- 5X reaction buffer : 1M potassium cacodylate, 125 mM Tris-HCl, 1.25 mg/ml bovine serum albumin; pH 6.6 (25°C)
- $CoCl_2$ solution : 25 mM cobalt chloride ($CoCl_2$)
- DIG-11-ddUTP : 1 mM digoxigenin-11-ddUTP (2'3'-dideoxyuridine-5'-triphosphate coupled to digoxigenin via an 11-atom spacer arm) in redistilled water.
- Glycogen solution : 20 mg/ml glycogen in redistilled water
- Terminal transferase : 50 U/µl terminal transferase in 200 mM potassium cacodylate, 1 mM EDTA, 200 mM KCl, 0.2 mg/ml bovine serum albumin; 50% (v/v) glycerol; pH 6.5

2. DIG luminescent detection kit **Boehringer Mannheim 1363514**

- Anti-digoxigenin-AP, Fab fragments
- CSPD, Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro) tricyclo [3.3.1.1^{3,7}] decan}-4-yl) phenyl phosphate
- Blocking reagent

3. Megaprime^{TM} DNA labelling system **Amersham RPN 1606**

- Primer solution : random nanamer primers in aqueous solution
- Labelling buffer : dATP, dGTP and dTTP in Tris-HCl, pH 7.5, 2-mercaptoethanol and $MgCl_2$
- Reaction buffer : 10X concentrated buffer containing Tris-HCl, pH 7.5, 2-mercaptoethanol and $MgCl_2$
- Enzyme solution : 1 U/µl DNA polymerase I Klenow fragment (cloned) in 50 mM potassium phosphate pH 6.5, 10 mM 2-mercaptoethanol and 50% glycerol

4.

5. Nick column **Pharmacia Biotech 52-2076-00**

- 0.9 X 2.0 cm gel bed containing Sephadex G-50 DNA grade in distilled water with 0.15% Kathon CG as preservative.

5. QIAGEN plasmid midi kit — Qiagen 12143

- Buffer P1 : 50 mM Tris-HCl, pH 8.0; 10 mM EDTA
- Buffer P2 : 200 mM NaOH, 1% SDS
- Buffer P3 : 3.0 M Potassium acetate, pH 5.5
- Buffer QBT : 750 mM NaCl; 50 mM MOPS, pH 7.0; 15% ethanol; 0.15% Triton X-100
- Buffer QC : 1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% ethanol
- Buffer QF : 1.25 M NaCl; 50 mM Tris-HCl, pH 8.5; 15% ethanol
- QIAGEN tips : The tips was regenerated by 5 ml buffer QF and soaked with 70% ethanol at 4 °C. A collection of tips was autoclaved at 121 °C at 1 $kgcm^{-2}$ and dried at 60 °C. The regenerated tips were kept at room temperature and ready for use.

6. AutoRead Sequencing Kit — Pharmacia XY-056-00-02

- A mix : 5 μM ddATP, 1mM dATP, 1mM dCTP, 1mM dTTP, 1mM c7dGTP, 50 mM NaCl and 40 mM Tris-HCl, pH 7.6
- C mix : 5 μM ddCTP, 1mM dATP, 1mM dCTP, 1mM dTTP, 1mM c7dGTP, 50 mM NaCl and 40 mM Tris-HCl, pH 7.6
- G mix : 5 μM ddGTP, 1mM dATP, 1mM dCTP, 1mM dTTP, 1mM c7dGTP, 50 mM NaCl and 40 mM Tris-HCl, pH 7.6
- T mix : 5 μM ddTTP, 1mM dATP, 1mM dCTP, 1mM dTTP, 1mM c7dGTP, 50 mM NaCl and 40 mM Tris-HCl, pH 7.6
- T7 DNA polymerase : 8 units/μl in 25 mM Tris-HCl, pH 7.5, 0.25 M NaCl, 3 mM DTT and 50 % glycerol
- Enzyme dilution buffer : 20 mM Tris-HCl, pH 7.5, 5 mM DTT, 10 μg/ml BSA and 5% glycerol
- M13 universal primer : 5'-fluorescein-d[CGA CGT TGT AAA ACG ACG GCC AGT]-3' in aqueous solution, 1.5 μM (2.1 pmol; 0.55 A_{260} unit/ml)
- M13 reverse primer : 5'-fluorescein-d[CAG GAA ACA GCT ATG AC]-3' in aqueous solution, 2.1 μM (2.1 pmol/μl; 0.42 A_{260} unit/ml)
- Annealing buffer : 1 M Tris-HCl, pH 7.6 and 100 mM $MgCl_2$
- Extension buffer : 304 mM citric acid, 324 mM DTT and 40 mM $MnCl_2$
- DMSO : Dimethyl sulfoxide
- Stop solution : 100 % deionized formamide and Dextran Blue 2000 (5 mg/ml)

A4. List of machine and equipment

ALF ^{TM} DNA Sequencer	Pharmacia
Bench-top microcentrifuge	Eppendorf 5415C
Bio-Dot microfitration apparatus	BioRad SF
Centrifuge	Beckman J2-M1
GeneQuant, RNA/DNA calculator	Pharmacia 80-2103-68
Dri-Bath	Thermolyne 17600
Hybridization chamber	Hybraid Midi Dual D110
Imaging Densitometer	BioRad GS-670
pH meter	Jenway 3010
Programmable thermal controller- Peltier effect	MJ Research PTC-100^{TM}
SpeedVac concentrator	Savant SC110
Ultracentrifuge	Beckman XL-80
X-Omatic cassette (regular screens)	Kodak